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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Neurofibromatosis type 1 (NF1) is a genetic disorder characterized by tumors comprised principally of Schwann cells lacking the neurofibromin gene. Our studies concentrated on the metabolic changes that occur in Schwann cells derived from NF1 tumors. We documented the following changes: aberrant expression of cell surface receptors, including c-Kit and PDGF, novel pathways stimulated by activation of these receptors leading to the prevention of apoptosis and increased calcium levels, abnormal secretion of prostaglandin, and subsequent activation of prostaglandin receptors, increased expression of prostaglandin receptors, and elevated cAMP. Developmental studies of c-Kit revealed the role for this cell surface receptor during development in preventing apoptosis. The overexpression of these receptors and subsequent changes in intracellular metabolism all contribute to the enhancement of the proliferative potential of Schwann cells, allowing increased tumor growth. These altered metabolic pathways provide new therapeutic targets for controlling the growth of Schwann cells in tumors in neurofibromatosis type 1.				
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A. Introduction

The subject of this investigation is Type 1 neurofibromatosis (NF1), which is an inherited disease that occurs at a frequency of approximately 1 in 3,000. Two types of tumors are evident in this disease; relatively benign neurofibromas, which occur as subcutaneous tumors, and neurofibrosarcomas (NFS), the more invasive and aggressive tumor types that can potentially be life-threatening. The major cell type that proliferates and forms either of these tumors is the Schwann cell. Our laboratory has obtained and developed a number of Schwann cell lines derived from neurofibrosarcomas and or neurofibromas, which we are currently using to study this disease. In addition, we have obtained benign Schwann Cell tumors (Schwannomas) from patients who are not affected by NF1 to serve as controls for the neurofibromatosis-derived Schwann cells. The purpose of this investigation is to understand the molecular mechanisms that are responsible for the abnormal proliferation of Schwann cells, which characterize NF1. In particular, we would like to understand how the absence of neurofibromin, leads to changes in intercellular signaling, which ultimately leads to a sustained proliferation of Schwann cells. Our approach to understand these changes is to use in vitro systems and Schwann cells that have been derived from either neurofibromas or neurofibrosarcomas. Our laboratory was fortunately in obtaining, from other researchers, a number of NF1-derived cell lines and in addition, we have developed several of our own. The control for all of these studies is normal human Schwann cells, which we believe to be the best control for establishing baseline transduction mechanisms of the non-diseased state. In addition, in order to understand how alterations in neurofibromin content could alter transduction pathways, several approaches were undertaken. First of all, one cell line, the STS26T contained no neurofibromin, but yet was abnormally proliferating and formed a type of control that was used in most of these studies. Secondly, we were able to establish a collaboration with Dr. Wade Clapp who has produced retroviral constructs containing the part of the neurofibromin molecule for which a known activity has been assigned, namely, the GTPase activating protein domain. The strategy in these studies is to transfect this domain back into the abnormal NF1-derived Schwann cells and observe subsequent changes in signal transduction and biochemistry. Taken as a whole, these studies as outlined in the body of this progress report, have given us new understanding of the metabolic changes that take place in neurofibromatosis cells.

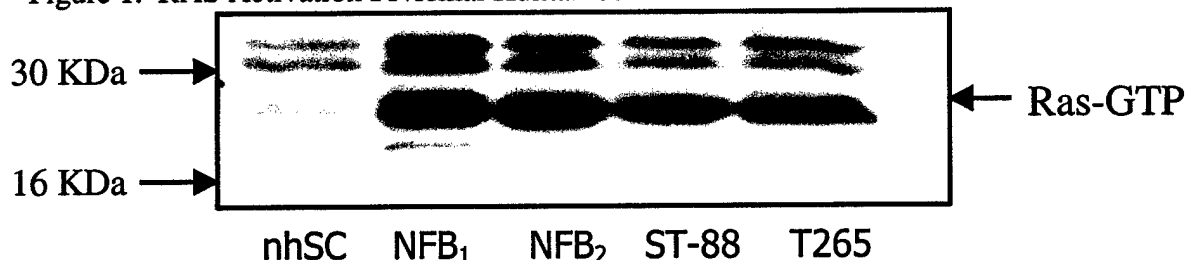
B. Body

This section of the progress report will include experimental methods, results and discussion in relation to the statement of work outlined in the award proposal. In this section of the proposal, we will outline the three major statements of work in the original proposal and the progress that has been made in accomplishing these goals. In addition, based on the outcomes of some of our investigations, the third statement of work was modified leading to a new statement of work and progress in achieving that goal is also outlined in the body of this report. There were three major statements of work and sub-aims within those statements. In order to be most comprehensive, we will give the overall statement of work and the progress that has been made toward accomplishing the goals of that statement.

First Overall Statement of Work: Effect of decreased neurofibromin expression on human Schwann cell proliferation

To accomplish this statement of work, it was necessary to first establish that other cell lines that we previously showed lacked neurofibromin, indeed had higher levels of Ras as expected. After consultation with other researchers in the field of neurofibromatosis, we realized that trying to decrease neurofibromin expression, as we originally proposed, would not be possible. Therefore, we took the opposite approach. That is, the consequence of decreased neurofibromin expression is increased Ras activity in the NF1-derived Schwann cells. In collaboration with Dr. Wade Clapp, we obtained a retroviral construct by which we could transfect the GAP related domain of neurofibromin into the NF1-derived cells. The predicted consequence of doing this would be a decrease in the levels of activated Ras as well as downstream events that were dependent on the expression of Ras. Therefore, prior to trying to decrease activated RAS levels in the NF1-derived Schwann cell lines, we established that the cells lacked neurofibromin and contained hyperactivated RAS relative to RAS activation in normal human Schwann cells. We have already established that the cell lines we will use in these studies lack neurofibromin (See Badache, two papers). We have also been successful in establishing two new cell lines from neurofibromas obtained from NF1 patients (NFB₁ and NFB₂). These cell lines have also been shown to lack neurofibromin (Gollapudi and DeVries, unpublished observations). We isolated normal human Schwann cells and using the "RAS-pull down" assay (described in the specific methods section of this proposal) determined the state of RAS activation in these cells. The results are shown in Figure 1 below.

Figure 1. RAS Activation I Normal Human Schwann cells and Schwann cell lines



Note that both the neurofibroma-derived and the ST-88 and T-265 cells (which are derived from malignant peripheral nerve sheath tumor cells) are hyperactivated with respect to normal human Schwann cells. It is interesting to note that the less malignant neurofibroma-derived cell

lines (NFB₁, NFB₂) also contain hyperactivated RAS. This is a key piece of data, since the cell lines with hyperactivated RAS are starting point for all the studies in this proposal.

The GAP-related domain (GRD) of neurofibromin was transfected into the T265 cell line (see Hiatt et al, 2001). The cells transfected with the construct were cloned and analyzed for the expression of the KT-3 epitope, which is incorporated into the construct for the GRD. The results are shown below.

Figure 2: – Western Blot analysis for KT-3 Epitope in Transfected T265 Cells

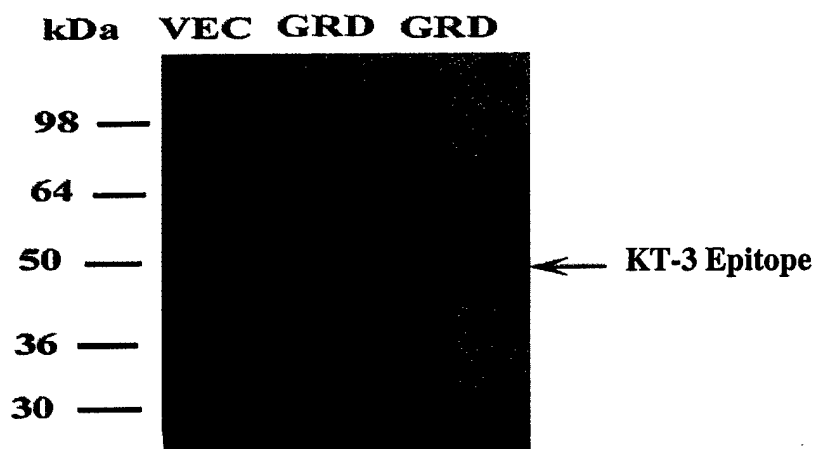


Figure 2: – Clones derived from T265 cells transfected with the GAP-related domain (GRD) of NF1 were analyzed by Western blotting for the KT-3 epitope, included in the transfected GRD. Molecular weights are shown to the left of the figure. VEC = cells transfected with plasma vector only, GRD= cells transfected with GAP-related domain.

Note the absence of the KT3 epitope in the T265 cells transfected with the vector only (VEC) and the presence of the epitope in the GRD transfected cells, confirming the expression of the GRD in these cells.

To further confirm the presence of the GAP-related domain, we analyzed the cells for the presence of the mRNA for the GRD in the transfected cells.

Figure 3: PCR Analysis for NF-GAP mRNA in Transfected T265 cells

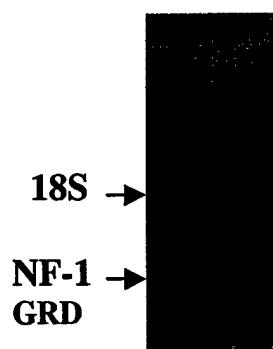


Figure 3. Clones from NF1 T265 GAP-domain (NF1 GRD) transfected T265 cells were grown to confluence, then RNA was extracted, reverse transcribed and analyzed by PCR using primers specific for the mRNA for NF1 GRD. Primers for 18S RNA were included as a positive control.

Note that the T265 cells transfected with the vector only did not express the mRNA for the GRD whereas the T265 cells transfected with the GRD strongly expressed the mRNA for GRD, further confirming the transfection of the GRD construct in these cells.

Although the GRD is present, it is important to show that it is active and alters intracellular metabolic pathways. At the time we developed the GRD-containing Schwann cell clones, we had not set up the "RAS pull down" assay. We have not yet had the opportunity to directly assay activated RAS. Therefore, we measured the effects of the GRD indirectly by measuring MAP kinase activity in the GRD transfected cells (see methods section of proposal) via Western blot analysis of activated phosphorylated MAP kinase. The results are shown below.

Figure 4: Effect of GRD on MAP kinase activity in Schwann cell lines

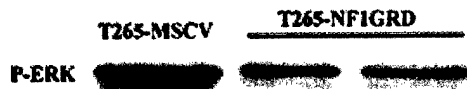


Figure 4: Cell lysates (25 µg) of T265 cells transfected with vector (T265-MSCV) or NF1 transfected with the NF1 GRD. We analyzed these cells for the phosphorylated form of MAP kinase (P-ERK).

Note that the levels of activated MAP kinase were decreased by at least twofold providing indirect evidence that RAS activity was decreased in the NF1 GRD transfected cells.

In summary, progress on this goal has been the establishment of several clones of GAP transfected cell lines. In addition, we now have, in our own hands, the tools to transfect this GAP domain into other cell lines that are currently being developed in the laboratory. The results are encouraging in that expected changes in events downstream from activated Ras are occurring. The obvious experiment that is still in progress is the determination of levels of Ras subsequent to transfecting the GAP related domains into the cells. These experiments are currently in progress (Gollapudi and DeVries, unpublished observations, paper in preparation).

Overall Task 2: Role of Kit expression in NF1-derived Schwann cell proliferation

Type 1 Neurofibromatosis (NF1) is characterized by the formation of neurofibromas, benign tumors composed mainly of Schwann cells, which can turn malignant to form neurofibrosarcomas. Neurofibromin, the protein product of the NF1 gene, is believed to act as a tumor suppressor, accelerating the conversion of the oncogene Ras to its inactive form. The absence of neurofibromin could therefore lead to higher Ras activity in Schwann cells, resulting in uncontrolled growth through a cascade of events not yet elucidated. We describe the abnormal expression of high levels of the Kit tyrosine kinase receptor in both NF1-derived Schwann cell lines and tissue, as compared to primary Schwann cells or schwannoma-derived cells. High levels of Kit expression in the neurofibrosarcoma-derived Schwann cells correlate with a decrease in neurofibromin expression. Using inhibitors of tyrosine kinase receptors, we found that proliferation of the neurofibrosarcoma-derived cells is dependent upon activation of a subclass of tyrosine-kinase receptors. The proliferation of these cells is not dependent upon an autocrine loop involving typical Schwann cell mitogens. Finally, the proliferation of the neurofibrosarcoma-derived Schwann cells can be increased by stimulation with Kit ligand.

These data implicate Kit as one of the components leading to the Schwann cell hyperplasia observed in NF1 (Badache, Muja and DeVries, 1998, included in Appendix).

Having established that Kit is overexpressed in the NF1-derived Schwann cells, we were interested to know the physiological consequences of that overexpression. In addition, we extended our studies to look at the expression of other growth factor receptors to see if this was a common theme among the NF1-derived Schwann cells. In our next study, we show that platelet-derived growth factor (PDGF) BB, and to a lesser extent fibroblast growth factor 2, are mitogenic for two neurofibrosarcoma-derived Schwann cell lines, but not for a Schwann cell line derived from a schwannoma (from a non-NF1 patient) or for transformed rat Schwann cells. Levels of expression of both PDGF receptor α and β are significantly increased in the two neurofibrosarcoma-derived cell lines compared to the non-NF1 Schwann cell lines. The level of tyrosyl-phosphorylated PDGF receptor β is strongly increased upon stimulation by PDGF BB. In comparison, only modest levels of tyrosyl-phosphorylated PDGF receptor α are observed, upon stimulation by PDGF AA or PDGF BB. Accordingly, PDGF AA is only a weak mitogen for the neurofibrosarcoma-derived cells by comparison to PDGF BB. These results indicate that the mitogenic effect of PDGF BB for the neurofibrosarcoma-derived Schwann cell lines is primarily transduced by PDGF receptor β . Neu differentiation factor β , a potent mitogen for normal Schwann cells, was unable to stimulate proliferation of the transformed Schwann cell lines, due to a dramatic downregulation of the erbB3 receptor. Therefore, aberrant expression of growth factor receptors by Schwann cells, such as the PDGF receptors, could represent an important step in the process leading to Schwann cell hyperplasia in NF1 (Badache and DeVries, 1998, included in Appendix).

In many cancer cells, molecules that play an important roles during development are inappropriately re-expressed and contribute to the tumor growth of those cells. Having established that c-Kit is overexpressed, as well as other growth factor receptors, we now attempted to understand the role of c-kit in normal Schwann cell physiology and how that role may be altered in the NF1-derived Schwann cells. Although stimulation of c-kit in these cells only slightly increased the basal Schwann cell proliferation (Badache et al, 1998a), we now investigate the relationship of c-kit and neurofibromin expression and the potential role of c-kit in Schwann cell development. Neurofibromin and c-kit are inversely regulated in rat sciatic nerves during development: c-kit is virtually absent in adult nerve whereas neurofibromin is strongly expressed in adult nerve. Normal adult human Schwann cells contain neurofibromin and do not express c-kit, while human Schwann cell lines derived from NF1 tumors express c-kit but do not express neurofibromin. In cultured neonatal rat Schwann cells, activation of c-kit by stem cell factor prevents programmed cell death via the activation of Akt but does not induce Schwann cell proliferation or differentiation. These results indicate an anti-apoptotic role for c-kit during Schwann cell development. Abnormal expression of this receptor in NF1-derived Schwann cells may contribute to tumor growth via inhibition of apoptosis (Dang and DeVries, 2002, included in Appendix).

In summary, these studies conclusively show, for the first time, that growth factor receptor overexpression (especially those for stem-cell factors and PDGF) are characteristic of cells that have lost neurofibromin. These receptors, when appropriately stimulated, may prevent cell death of the Schwann cells (c-Kit receptor) or contribute to the overall proliferation (PDGF receptor). Further studies to understand how the loss of neurofibromin leads to the overexpression of these receptors are currently underway.

Overall Task 3: Effect of simultaneous Kit expression and neurofibromin alteration on human Schwann cell proliferation

In our original proposal, we planned to overexpress Kit using a dominant negative construct while blocking neurofibromin to mimic the conditions we saw in the NF1-derived Schwann cells. However, attempts at blocking neurofibromin expression in human Schwann cells have been completely futile (D. Muir personal communication), and we were discouraged by the fact that kit played only an anti-apoptotic role and not a strong proliferative role in the NF1-derived Schwann cells. Therefore, we modified this task to continue to make progress on understanding the transduction mechanisms in transformed Schwann cells as shown in Overall Task 4. Recently, we have attempted to elevate Ras in normal human Schwann cells by blocking the removal of a palmityl group from Ras so that it remains in an inactivated state. Preliminary results in this regard are encouraging, since we have found an increased level of activated Ras (Farrer, Farrer and DeVries, unpublished observations). In addition, a most exciting recent finding is that with the increased Ras activation, we began to see c-Kit expression in the normal human Schwann cells. Further studies in this regard are underway.

Overall Task 4: Role of Altered Intracellular Metabolism of NF1-derived Schwann cells in the etiology of neurofibromatosis Type I.

Neurofibromatosis Type 1 tumors are highly vascularized and contain Schwann cells with hyperactivated Ras. In vitro, the NF1-derived neurofibromin efficient Schwann cells have an angiogenic profile, which favors angiogenesis and sustains the growth of the NF1-derived tumors. This study examines the relationship of the activation state of cultured Nf-1 derived Schwann cells and normal human Schwann cells. Western blot analysis of normal human Schwann cells revealed low expression of angiogenic vascular endothelial growth factor (VEGF) as well as low expression of the anti-angiogenic pigment epithelium derived factor (PEDF). Relative to normal human SC, NF1-derived Schwann cells have increased Ras activity and a three-fold increase in VEGF expression. Surprisingly, PEDF was also expressed in the NF1-derived Schwann cells at approximately the same level as VEGF expression. Using a murine stem-cell virus, we introduced the GAP-related domain of neurofibromin into the NF1-derived Schwann cells to reduce the level of activated Ras. Relative to the untreated NF1-derived Schwann cells, the Schwann cells expressing the GAP-related domain expressed about one half the VEGF but twice the PEDF. We conclude that decreasing the Ras activity in NF1-derived Schwann cells will not only decrease proliferation but also slow tumor angiogenesis due to the decreased expression of angiogenic and increased expression of anti-angiogenic factors (Thomas and DeVries, unpublished observations, paper in preparation, included in Appendix).

The preliminary data that supports the results of the previous paragraph is shown in the figures below. Figure 5 demonstrates VEGF expression in Normal and NF1-derived Schwann cells. Note that relative to normal human Schwann cells, both the T265 and ST88 NF1 Schwann cells as well as the 90-8 show increased expression of VEGF. The expression of both the angiogenic VEGF and the anti-angiogenic PEDF are demonstrated in Figure 6 below.

Figure 5 – VEGF Expression in Normal and NF1-Derived Schwann Cells

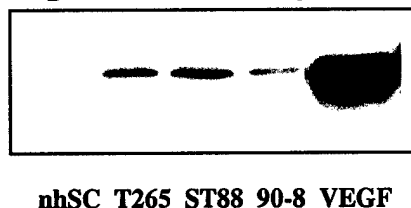


Figure 5. Western blot analysis of VEGF in cell lysate from normal human Schwann cells (nhSC), the MPNST-derived Schwann cell lines T265 and ST88, and the neurofibroma-derived Schwann cell line 90-8. VEGF expression is the highest in the malignant Schwann cell lines, intermediate in the benign neurofibroma cell line, and barely detectable in nhSC.

Note that the T265 Parent cell line, as previously demonstrated, has increased expression of VEGF. However, two of the GAP related domain transfected parent cell lines, termed GRD7 and GRD9 show markedly decreased VEGF expression. Concomitantly, these GAP domain transfected clones show increased expression of the anti-angiogenic PEDF. These results support the view that the angiogenic profile, as the extent of activated Ras, is decreased. The angiogenic potential of the cells is reduced.

Figure 6 – Altered VEGF and PEDF Expression in NF1-derived Schwann Cells after NF1-GRD Transfection

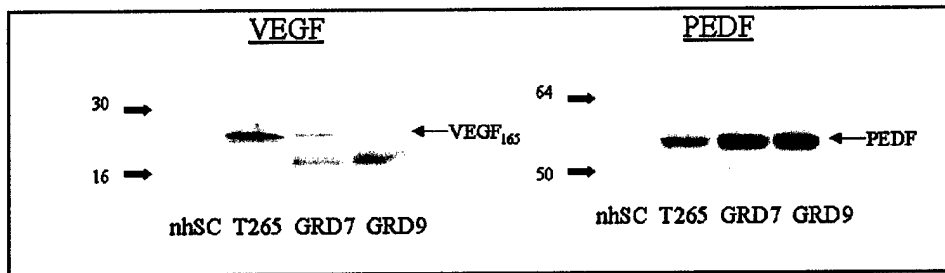


Figure 6. Western blots comparing VEGF and PEDF expression in lysates from normal human Schwann cells (nhSC), the MPNST-derived cell line T265, and two T265 clones transfected with the NF1-GRD (GRD7 and GRD9). VEGF expression is increased in T265 compared to nhSC, but VEGF expression decreases with transfection of the NF1-GRD in clones GRD7 and GRD9. It is interesting that GRD7 and GRD9 also express what may be a lower molecular weight isoform of VEGF. PEDF expression was not detected in nhSC using a commercially available antibody but is expressed in T265. PEDF expression was further increased due to NF1-GRD transfection as seen in GRD7 and GRD9.

Although the previous data demonstrated changes in the expression of these angiogenic and anti-angiogenic factors in whole cell lysates, the interesting question is what happens to the expression in media? The results of analysis for VEGF in medium is shown in Figure 7 below.

Figure 7: NF1-derived Schwann cells transfected with the GAP domain secrete less VEGF

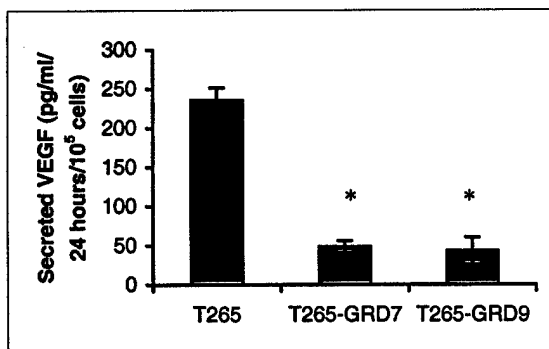


Figure 7. ELISA analysis of media conditioned by the MPNST-derived cell line T265 and two NF1-GRD transfected T265 clones, T265-GRD7 and T265-GRD9. VEGF concentration significantly decreased in media conditioned by the NF1-GRD expressing cells T265-GRD7 and T265-GRD9.

As a whole, these results demonstrate that when the GAP related domain of neurofibromin is transfected into NF1-derived Schwann cells, the angiogenic properties of those

cells are greatly altered. This, for the first time, gives us some understanding of how the loss of neurofibromin may affect the overall metabolism of Schwann cells leading to tumor growth.

Other metabolic changes in NF1-derived Schwann cells were also investigated, particularly how signal transduction occurred via PDF receptors that were overexpressed in the NF1-derived Schwann cells. We were intrigued by the finding of the overexpression of PDGF receptors and what transduction mechanisms may be activated by PDGF in these cells. Therefore, the next study was undertaken.

The growth-factor receptor expression levels and signal transduction pathways of NF1 Schwann cells and normal adult Schwann cells were compared to better understand the molecular basis of hyperplasia in NF1. The PDGF receptor levels are higher in NF1 tumors than the PDGF receptor levels found in normal adult peripheral nerves. However, the PDGF receptor levels are similar in normal adult and NF1 Schwann cells. Signal transduction downstream of the PDGF receptor also was studied in the two cell types. Erk and Akt of the PI3K pathway are phosphorylated in response to PDGF BB in both cell types. However, PDGF stimulated changes in cellular calcium levels and revealed increased intracellular calcium levels in NF1 Schwann cells but no change in calcium was observed in normal adult Schwann cells. The calcium response in the NF-1 Schwann cells originated in the internal compartment of the cell rather than from its extracellular compartment. The calcium downstream effector calmodulin kinase II (CAMKII) is phosphorylated in response to PDGF BB in NF-1 Schwann cells. In addition, the growth of NF1 Schwann cells stimulated by PDGF BB is decreased with the CAMKII inhibitor, KN62. These results indicate that the aberrant activation of the calcium signaling pathway in NF-1 Schwann cells contributes to the formation of NF1 tumors (Dang and DeVries, 2002, included in appendix).

At this point, we realized that cells that had lost neurofibromin had altered angiogenic profiles and altered calcium metabolism. Another important metabolic property of cells is their ability to migrate. Loss of neurofibromin may well influence changes in the migratory potential of NF1-derived Schwann cells.

We next found that PDGF BB strongly stimulates the migration of neurofibrosarcoma-derived Schwann cells in a Boyden-like chamber assay. The effect of PDGF BB is not simply chemokinetic, but chemotactic since it was largely dependent on the presence of a gradient of PDGF BB. PDGF AA, fibroblast growth factor 2 or stem-cell factor (Kit ligand), previously shown to be mitogens for neurofibrosarcoma cells, are poor chemoattractants. PDGF BB also stimulates neurofibrosarcoma-derived cell invasion of a reconstituted matrix. NF1-derived cells secrete increased levels of matrix metalloproteinases 2 and 9 relative to normal Schwann cells, which could explain their high invasive potential. Results of this study indicate that PDGF BB, through its mitogenic and chemotactic properties, could contribute to the malignant phenotype of neurofibrosarcoma-derived Schwann cells (Badache and DeVries, 2001, included in Appendix).

Since we had already demonstrated changes in the PDGF and c-Kit receptor expression, we next investigated whether receptors for prostaglandin, such as the prostaglandin derivative thromboxane A₂, was altered in the NF1-derived Schwann cells. Previous reports have demonstrated the presence of functional thromboxane A₂ (TXA₂) receptors in both astrocytes and oligodendrocytes. In these experiments, the presence and function of TXA₂ receptors in primary rat Schwann cells (rSC) and a neurofibrosarcoma-derived human Schwann cell line (T265) was investigated. Immunocytochemical and immunoblot analyses using polyclonal anti-TXA₂ receptor antibodies demonstrate that both cell types express TXA₂ receptors. To test the functionality of TXA₂ receptors in these cells, we monitored intracellular calcium levels following receptor stimulation. Treatment with the stable TXA₂ mimetic U46619 (10 μ M) did not stimulate intracellular calcium mobilization in rSC whereas T265 cells demonstrated a

calcium response that was inhibited by prior treatment with TXA₂ receptor antagonists. U46619 also stimulated CREB phosphorylation on Ser-133 in T265 cells and, to a lesser extent, in rSC. To identify the mechanism of CREB phosphorylation in rSC, we monitored intracellular cAMP levels following U46619 stimulation. Elevated levels of cAMP were detected in both rSC (20-fold) and T265 cells (15-fold). These results demonstrate that TXA₂ receptor activation stimulates CREB phosphorylation in T265 cells, possibly by a calcium- and/or cAMP-dependent mechanism. In contrast, TXA₂ receptor activation in rSC stimulates increases in cAMP and CREB phosphorylation but does not elicit changes in intracellular calcium (Muja and DeVries, 2001, included in Appendix).

Since we had noted changes in a prostaglandin receptor in these cells, we next investigated the potential role of prostaglandin metabolism, which in turn could be linked to elevation of cAMP in the NF1-derived Schwann cells. We now report that NF1 Schwann cells have two-fold higher cAMP levels than normal human adult Schwann cells. PCR analysis of normal adult human Schwann cells reveals adenylyl cyclase (AC) mRNA for types III, IV, and IX. Similar analysis of NF1 Schwann cells shows all the isoforms in normal adult human Schwann cells, but additionally, the NF1 Schwann cells express AC mRNA of types II, V, VII, and VIII. Increased cAMP levels may be the results of prostaglandins secreted by Schwann cells themselves. In support of this view, we find that NF1 Schwann cells express higher levels of cPLA2 and Cox-2 than control cells. PCR analysis reveals that NF1 Schwann cells express mRNA for EP2 and EP4 prostaglandin receptors while normal human Schwann cells only express the EP2 receptor. Interestingly, the addition of exogenous prostaglandins to NF1 Schwann cells induced further increases of cAMP levels and also induces the proliferation of NF1 Schwann cells. The proliferation of NF1 Schwann cells in response to PDGF BB (was) decreased by Cox-2 and PKA inhibitors. These results are consistent with the view that aberrant cAMP signaling and elevated prostaglandin metabolism in NF1 Schwann cells contribute to tumor formation in NF patients (Dang and DeVries, in preparation, included in Appendix).

These results are consistent with the view that hyperactivation of Ras leads to hyperactivation of MAP kinase. In turn, this leads to increased phospholypase A2 activity. The phospholypase A2, then releases the primary substrate for prostaglandin, which is arachidonic acid, leading to abnormal secretion of prostaglandins. Taken as a whole, our laboratory has, for the first time, established a number of key metabolic changes that occur in NF1-derived Schwann cells that lack neurofibromin.

C. Key Research Accomplishments

- Several clones of cells transfected with the GAP domain of neurofibromin have been established.
- The hyperactivation of Ras in NF1-derived Schwann cells has been verified relative to regular normal human Schwann cells.
- It has been established that transfection of the GAP related domain into NF1-derived Schwann cells leads to decreased MAP kinase activity.
- The tyrosine kinase receptor, c-Kit, is present in high concentrations in NF1-derived Schwann cells.
- The platelet-derived-growth-factor receptor (PDGF) is overexpressed in NF1-derived Schwann cells.
- Activation of the PDGF receptor by PDGF leads to increased proliferation of the NF1-derived Schwann cells.
- The receptor for Neu differentiation factor is lacking in NF1-derived Schwann cells.
- C-Kit appears to play an important anti-apoptotic role in normal Schwann cell development.

- Activation of c-Kit in neonatal Schwann cells leads to prevention of apoptosis.
- Activation of c-Kit receptor by stem-cell factor leads to activation of the Akt, anti-apoptotic pathway.
- Transfection of the GAP related domain into NF1-derived Schwann cells decreases the angiogenic potential of NF1-derived Schwann cells.
- Transfection of the GAP related domain into NF1-derived Schwann cells increases the anti-angiogenic PEDF expression while simultaneously decreasing VEGF expression; the decreased VEGF expression is reflected in the lower secretion of VEGF by these cells.
- Activation of the PDGF receptor leads to increased intracellular calcium in the case of NF1-derived Schwann cells, while it is completely refractory in normal human Schwann cells.
- Activation of the PDGF receptor in NF1-derived Schwann cells leads to increased migration of the NF1-derived Schwann cells.
- NF1-derived Schwann cells express high levels of thromboxane A2 receptor.
- NF1-derived Schwann cells contain elevated cAMP as well as elevated expression of prostaglandin receptors and secrete prostaglandins.

D. Reportable Outcomes

Presentations:

1999 National Neurofibromatosis Foundation Consortium-Molecular Biology of NF-1 & NF-2.
Presentation: Altered Growth Factor Receptor Expression in NF Cells."

Medical College of Virginia- Department of Biochemistry and Molecular Biophysics,
"Molecular Mechanisms of Schwann Cell Proliferation in Neurofibromatosis," February 21,
2000.

University of Chicago, Kennedy Institute, "Molecular Mechanisms of Schwann Cell
Proliferation in Neurofibromatosis," November 16, 1999

Papers Published:

Muja, N., Blackman, SC, LeBreton, GC. and DeVries, GH (2001). Identification and functional characterization of thromboxane A2 receptors in Schwann Cells. *J. of Neurochem*, August; 78(3);446-56.

Badache, A. and G.H. DeVries (1998) Neurofibrosarcoma-derived Schwann cells overexpress PDGF receptors and are induced to proliferate by PDGF BB. *J. Cell. Phys.*, 177:334-342.

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Dang, I. and DeVries, G.H. NF1 Schwann cells have elevated cAMP levels and increased, secretion of prostaglandin PGE₂

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Development of cell lines:

- GAP transfected cell lines
- New neurofibromatosis-derived cell line

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E. Conclusions/So What?

What have we learned about neurofibromatosis from the studies supported by this proposal? As a result of these *in vitro* studies, we have gained a new appreciation of the metabolic changes that occur in Schwann cells of individuals affected by this disease. Schwann cells are the major cell type, which make up the tumors found in neurofibromatosis. Ongoing *in vitro* and *in vivo*

investigations are aimed at understanding why these cells divide abnormally, form benign and malignant tumors in this disease. As a result of our investigations, we now understand that the loss of the gene product of neurofibromin, which has been identified as the causative gene in this disease, results in significant alterations in the metabolism of these cells, which tilts the balance toward proliferation. It should be noted that in all cases, the metabolic changes that were documented were always with reference to normal human Schwann cells. This type of control has not always been used in other laboratories.

What metabolic changes have we documented? First of all, several types of cell-surface receptor, which when activated with the appropriate growth factor lead to proliferation, are elevated in NF1-derived Schwann cells. These cell surface receptors include c-Kit, PDGF, and prostaglandin. Having these receptors expressed is like having a loaded revolver in that any encounter with the appropriate activating molecule will trigger a proliferative response in these cells. Our investigation has uncovered a novel and previously unsuspected metabolic short-circuit that is present in the NF1-derived Schwann cells. The loss of the neurofibromin gene product leads to elevated levels of a transducer molecule, known as Ras. In turn, downstream molecules that receive signaling from this molecule also are activated. We have discovered that one of these downstream signaling molecules, MAP-Kinase, is hyperactivated in the NF1-derived Schwann cells. In turn, this results in increased activity and expression of an enzyme, known as phospholypase A2, which releases a molecule that is the starting point for a metabolic pathway ultimately resulting in elevated cAMP levels. Elevated cAMP recently has been recognized as an important consequence of the loss of the neurofibromin gene. However, currently there is no adequate explanation as to how this cAMP becomes elevated. As a result of our investigation, we now propose that activated Ras leads to activated MAP-Kinase, which in turn activates phospholypase A2 leading to prostaglandin secretion. We have documented increased prostaglandin secretion by the NF1-derived Schwann cells. In turn, the prostaglandins stimulate prostaglandin receptors, which we have discovered to be expressed at abnormally high levels and in abnormal varieties on the surface of the NF1-derived Schwann cells. It is well known that activation of these receptors by prostaglandin leads to elevated cAMP. Thus, our studies provide, for the first time, a link between the loss of the neurofibromin gene and elevated cAMP. It should be appreciated that elevated cAMP will tilt the metabolic balance of the cell in favor of proliferation.

Not only are the cell-surface receptors expressed in higher levels and in increased varieties, but when activated with the appropriate molecule, there are inappropriate metabolic responses. For example, we have found that activation of the PDGF receptor leads to increasing in intracellular calcium. This is clearly not the case with normal human Schwann cells. In addition, activation of other of these cell-surface receptors leads to activation of an intracellular pathway, which prevents "programmed cell death" and allows the cells to survive. Activation of the PDGF receptor also allows the cell to secrete certain kinds of enzymes that allow it to invade new tissue and promote tumor growth. In summary, the combination of abnormal cell-surface receptor expression and activation of abnormal pathways all favor the proliferative state for the NF1 Schwann cells.

Another exciting finding from these studies, which has been presented in abstract form but not yet published, is that the metabolism of Schwann cells is altered so that there is overexpression of secreted factor which favor blood vessel growth. The process of blood vessel growth, or angiogenesis, is known to be an important promoter of tumor growth, since the additional cellular mass requires additional blood vessels to provide sufficient oxygen for the growing tissue. The change to a "pro-angiogenic or angiogenic state" from a relatively neutral

state with respect to angiogenesis as is the case with normal human Schwann cells, also favors the type of threatening tumor growth, which is a major problem of neurofibromatosis.

The most exciting aspect of these investigations is not only the newly documented metabolic changes, but the availability of new therapeutic targets for intervention in this problematic disease. Our results suggest that by blocking the hyperactivation of Ras, many of these metabolic consequences can be avoided. Our results also suggest that the production of angiogenic factors is an important aspect of tumor growth, and as in many other cancers, targeting angiogenesis may be an effective therapeutic approach. Indeed, our laboratory currently is attempting to use molecular biological strategies to decrease activation of Ras by blocking the addition of molecules to the Ras, which allow it to bind to a membrane and become activated. Initial studies in this regard are promising. We are confident, as a result of these investigations, that our laboratory has contributed significant new information to our understanding of the etiology of neurofibromatosis.

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Appendices

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Identification and functional characterization of thromboxane A₂ receptors in Schwann cells

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Abstract

Previous reports have demonstrated the presence of functional thromboxane A₂ (TP) receptors in astrocytes and oligodendrocytes. In these experiments, the presence and function of TP receptors in primary rat Schwann cells (rSC) and a neurofibrosarcoma-derived human Schwann cell line (T265) was investigated. Immunocytochemical and immunoblot analyses using polyclonal anti-TP receptor antibodies demonstrate that both cell types express TP receptors. Treatment with the stable thromboxane A₂ mimetic U46619 (10 μM) did not stimulate intracellular calcium mobilization in rSC, whereas T265 cells demonstrated a calcium response that was inhibited by prior treatment with TP receptor antagonists. U46619 also stimulated CREB phosphorylation

on Ser133 in T265 cells and, to a lesser extent, in rSC. To identify potential mechanisms of CREB phosphorylation in rSC, we monitored intracellular cAMP levels following U46619 stimulation. Elevated levels of cAMP were detected in both rSC (20-fold) and T265 (15-fold) cells. These results demonstrate that TP receptor activation specifically stimulates CREB phosphorylation in T265 cells, possibly by a calcium- and/or cAMP-dependent mechanism. In contrast, TP receptor activation in rSC stimulates increases in cAMP and CREB phosphorylation but does not elicit changes in intracellular calcium.

Keywords: cAMP, neurofibromatosis type 1, peripheral nerve, Schwann cell, thromboxane A₂, Wallerian degeneration. *J. Neurochem.* (2001) **78**, 446–456.

Thromboxane A₂ (TXA₂) is a labile lipid mediator which is synthesized by the sequential metabolism of arachidonic acid by cyclooxygenase and thromboxane synthase (Smith 1992; Halushka *et al.* 1995). Thromboxane A₂ is produced following the activation of a variety of cell types. The subsequent release of TXA₂ into the extracellular environment potentially stimulates platelet aggregation, smooth muscle constriction and hypertrophy, and cellular proliferation (Vane *et al.* 1998). The abnormal production and release of TXA₂ has been implicated in a wide variety of thrombotic and vasospastic disorders (FitzGerald 1991).

The receptor for TXA₂ has been purified, cloned and sequenced (Hirata *et al.* 1991; Kim *et al.* 1992; Nusing *et al.* 1993). The sequence of the gene indicates that the receptor is a member of the heptahelical superfamily of G protein-coupled receptors. Human TXA₂ receptor (TP) is encoded by a single gene on chromosome 19p13.3, which can be alternatively spliced to produce two variants of exon 3 encoding the C-terminal tail, TPα and TPβ (Raychowdhury

et al. 1994; Miggin and Kinsella 1998). The different C-terminal regions have been shown to influence the mechanism and kinetics of receptor desensitization (Yukawa *et al.* 1997; Walsh *et al.* 2000) and internalization (Parent *et al.* 1999). Thromboxane receptors have been shown to couple to multiple heterotrimeric G-proteins including G_q

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Abbreviations used: CREB, cyclic AMP response-element binding protein; DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's modified phosphate-buffered saline; Fura-2AM, fura-2 acetoxymethyl ester; G protein, GTP-binding protein; HEL, human erythroleukemia cell line; HBSS, Hank's balanced salt solution; NaF, sodium fluoride; NDF, Neu-differentiation factor; rSC, primary rat Schwann cells; SMP, solubilized platelet membranes; TP, thromboxane; TXA₂, thromboxane A₂; TXB₂, thromboxane B₂; U46619, 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin F_{2α}

(Knezevic *et al.* 1993; Allan *et al.* 1996), G_{12/13} (Offermanns *et al.* 1994; Allan *et al.* 1996; Djellas *et al.* 1999) and G_i (Gao *et al.* 2001; Ushikubi *et al.* 1994). Also, TP α couples to a novel high molecular mass G protein, G_h (Vezza *et al.* 1999). Activation of TP receptors has been shown to modulate intracellular cAMP levels ([cAMP]_i) in platelets and transfected Chinese hamster ovary and HEK293 cells (Hirata *et al.* 1996; Walsh *et al.* 1998; Cracowski *et al.* 2000). To date, however, there has been no demonstration of the TP receptor directly coupling to G_s.

In addition to their classical identification in platelets and smooth muscle cells, TP receptors have also been described in astrocytes (Nakahata *et al.* 1992), myelinated fiber tracts (Borg *et al.* 1994) and oligodendrocytes (Blackman *et al.* 1998). The presence of TP receptors in these cells suggests that these receptors may be involved in normal or abnormal CNS physiology. Microglia (Minghetti and Levi 1995) and astrocytes (Pearce *et al.* 1989; Bruner and Murphy 1993) have been shown to release TXA₂ following activation by bacterial lipopolysaccharide and ATP, respectively. Thus, the elevation in extracellular pyrimidine nucleotides (Langley and Pearce 1998) and microglial activation seen following CNS tissue damage may lead to the release of TXA₂ and the subsequent autocrine or juxtacrine activation of TP receptors in the CNS.

In comparison, the presence and function of TP receptors in peripheral nerve physiology have not been investigated. Evidence supporting the potential existence of TP receptors stems from the finding that macrophages, which are recruited to the sites of peripheral nerve injury to remove debris and form an environment that is conducive to nerve regeneration (Beuche and Friede 1986; Goodrum *et al.* 1994; Brück 1997), release inflammatory cytokines and lipid mediators such as prostaglandins and TXA₂ (Rothwell and Hopkins 1995; Brock *et al.* 1999). Evidence also exists for the endogenous production of thromboxane by Schwann cells (Constable *et al.* 1994). Given that functional TP receptors have been identified in CNS glia and that sources for thromboxane exist in the PNS, we hypothesized that functional TP receptors were also present in Schwann cells, which are the myelin-forming cells of the PNS. Thus, in these experiments, we used primary Schwann cells isolated from neonatal rat sciatic nerve (rSC) and a human Schwann cell line (T265) to determine whether Schwann cells expressed functional TP receptors.

Materials and methods

Isolation and culture of primary rat Schwann cells

Primary rSC were cultured from neonatal rat sciatic nerves as described by Brockes *et al.* (1979). Sciatic nerves from 2-day-old Sprague-Dawley rat pups were extracted and digested enzymatically with 0.3% collagenase (Serva) in serum-free Dulbecco's modified Eagle's medium (DMEM) with low glucose and 10 mM

HEPES for 3 h at 37°C. Following digestion, the cells were centrifuged at 100 g for 5 min, resuspended in 4–5 mL of low-glucose DMEM (1.0 g/L glucose) supplemented with 3.7 g/L NaHCO₃, 10 µg/mL gentamicin and 5% fetal calf serum. Cells were dispersed (1 mL/dish) into 100-mm culture dishes containing an additional 9 mL of the same medium. Cells were maintained in a humidified incubator at 37°C in the presence of 5% CO₂ and 95% air.

The following day, adherent cells were rinsed with calcium- and magnesium-free Dulbecco's modified phosphate-buffered saline (D-PBS) to dilute divalent cations in preparation for differential adhesion. The rinse media was aspirated and fresh D-PBS (4 mL) supplemented with 0.2% EDTA was added to the cells. After 1 min, the culture dishes were transferred to the stage of an inverted microscope. Under visual inspection, the side of the culture dish was tapped gently until the majority of the primary rSC was dislodged. Excess incubation in collecting media, as well as excess tapping, increased the release of fibroblasts from cultureware. Furthermore, rSC directly associated with fibroblasts were difficult to dislodge using this technique and were omitted to minimize fibroblast contamination. Medium containing detached primary rSC was collected and the cells were concentrated by centrifugation (100 g, 5 min) in 15 mL centrifuge tubes containing 4–5 mL of low-glucose DMEM to improve pellet formation. The supernatant was discarded and the cell pellet was resuspended in low-glucose DMEM for cell distribution to cultureware. Cultures obtained using this method were estimated to consist of 97–99% primary rSC according to their phase bright, narrow, bipolar morphology under microscopic view. In some cases, primary Schwann cells cultures were treated with 10 µM cytosine β-D-arabinofuranoside (Sigma, St Louis, MO, USA) for 24–48 h to eliminate any remaining fibroblasts.

Culture of T265 cells

A human Schwann cell line (T265) established from a neurofibrosarcoma isolated from an individual with neurofibromatosis type 1 was maintained by continuous culture in high-glucose DMEM (4.5 g/L glucose) supplemented with 5% fetal calf serum, 3.7 g/L NaHCO₃ and 10 µg/mL gentamicin (Badache *et al.* 1998). T265 cells were split (1 : 5 dilution) weekly using D-PBS supplemented with 0.2% EDTA and 0.0025% acetylated bovine trypsin. Cells used in this study were between passages 18 and 22.

Immunoblot analysis

Immunoblot analysis of phosphorylated CREB (Ser133) was performed according to the procedure of Tabernero *et al.* (1998) with some modifications. Cells were seeded onto six-well culture clusters at a density of 1 × 10⁶ cells/well and serum deprived overnight. Cells were treated with low-glucose DMEM (primary rSC) or high-glucose DMEM (T265) containing 10 µM of the stable TP receptor agonist U46619, 200 µM 3-isobutyl-1-methyl-xanthine, and 1 mM sodium fluoride (NaF) for 0, 5, 10, 15, 30 or 60 min. Immediately following incubation with agonist, 200 µL of lysis buffer [5 mM Tris-HCl, 10% glycerol, 1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 2 mM EGTA, 1 mM NaF and 10 µg/mL leupeptin] was added and the cell lysates were collected from the culture plates using a cell scraper and placed on ice. Lysates were boiled for 10 min and centrifuged for 1 min at 500 g. Twenty micrograms of protein was loaded in each lane of a 4–20% SDS-polyacrylamide gel (Novex, Carlsbad, CA, USA). Proteins

were resolved electrophoretically and then transferred to a poly(vinylidene) difluoride (PVDF) membrane. Membranes were incubated in PBS containing 4% milk (PBS-MLK) for 30 min and transferred to a solution of PBS-MLK containing rabbit anti-phosphorylated CREB Ser133 (1 : 1000 dilution, Upstate Biotechnology, Lake Placid, NY, USA) for 16 h at 4°C. Poly(vinylidene) difluoride membranes (DuPont, NEN, Boston, MA, USA) were rinsed in PBS (3 × 5 min) and incubated in PBS-MLK containing horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (1 : 5000 dilution, Transduction Laboratories, Lexington, KY, USA) for 1 h at room temperature (22°C). Membranes were rinsed as above and developed using Kodak BioMax II film and Super Signal chemiluminescent reagent (Pierce, Rockford, IL, USA).

Immunoblot analysis of TP receptor expression in rSC and T265 cells was performed as described in detail previously (Blackman *et al.* 1999) using a polyclonal antibody raised against a decapeptide sequence (HAALFEWHAV; residues 89–98) from the first extracellular loop of the receptor protein (Borg *et al.* 1993, 1994). Twenty micrograms of cell lysates was prepared and resolved as described above for phosphorylated CREB. Primary (1 : 1000) and secondary (1 : 5000) antibodies were diluted in blocking buffer containing 0.05% Tween-20. Primary and secondary antibody solutions were incubated with PVDF membrane for 1 h on a platform shaker at room temperature. Protein extracts of solubilized human platelet membranes (SMP, 10 µg) and a human erythroleukemia cell line (HEL, 20 µg) were used as positive controls for TP receptor protein expression (Mayeux *et al.* 1989; Borg *et al.* 1994; Allan *et al.* 1996).

Immunocytochemistry

Immunocytochemical analyses of TP receptor expression in primary rSC and the T265 cell line were performed as described previously (Blackman *et al.* 1999) with some modifications. Cells were grown on 12-mm cover glasses and fixed with cold methanol at –10°C for 5 min. Non-specific immune binding sites were blocked by incubation with 10% normal goat serum diluted in PBS (PBS/NGS) for 1 h. Cells were then incubated with primary antibody raised against a peptide sequence from the first extracellular loop of the TP receptor (P2Ab; 1 : 50 in PBS/NGS) for 1 h. In some cases, immunocytochemistry was performed using a primary antibody raised against the purified TP receptor protein (TxAb; diluted 1 : 50 in PBS/NGS). Following a PBS wash (3 × 5 min), cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted (1 : 100) in PBS/NGS for 45 min. Cells were then washed using PBS and mounted using FluoroGuard (Vector Laboratories, Burlingame, CA, USA). Fluorescence was visualized using a Jenaval microscope equipped for epifluorescence. Immunocytochemical detection of phosphorylated CREB (Ser133) in the T265 cell line was performed as described previously (Tabernero *et al.* 1998) except for the use of VIP (Vector Laboratories, Burlingame, CA, USA) as an horseradish peroxidase substrate.

Radioligand binding assay

Binding of [³H]SQ29,548 to intact primary rSC and T265 cells was evaluated in confluent monolayers of cells cultured in six-well plates. Briefly, cells were washed gently (2 × 1 mL) with warm phenol red-free Hank's balanced salt solution (HBSS). The binding

reaction took place in a final volume of 1 mL of HBSS. Following 5 min incubation at room temperature with either vehicle (total binding) or 20 µM unlabeled SQ29,548 (non-specific binding), the cells were incubated for 30 min at room temperature on an orbital shaker with various concentrations of [³H]SQ29,548 (0.625–20 nM). At the end of the incubation period, the cells were washed quickly (2 × 2 mL) with ice-cold HBSS (wash time < 10 s). The monolayers were solubilized by adding 1 mL of 0.5 N NaOH/0.5% SDS and incubating on an orbital shaker for 5 min. The cell lysate was neutralized by adding 3 N HCl and a sample was retained for protein concentration analysis using the BCA assay (Pierce, Rockford, IL, USA). The receptor-bound radioligand was measured by liquid scintillation spectrometry. Radioactivity counts were normalized to the measured protein concentration. Specific binding was calculated as the difference between binding measured in the absence and in the presence of 20 µM unlabeled SQ29,548. Analysis of radioligand binding data was performed using GRAPHPAD PRISM 3.0 (GraphPad Software). Saturation curves were subjected to non-linear regression analysis. Curves were fit to both one-site and two-site bindings and were compared with an *F*-test. *K_D* and *B_{max}* were determined from the best fit equation.

Digital fluorescence ratio imaging of intracellular calcium using fura-2 acetoxymethyl ester

Cells were seeded onto 25-mm glass coverslips in low-glucose DMEM (primary rSC) or high-glucose DMEM (T265 cells) at 37°C in the presence of 5% CO₂ for 6 h and then serum deprived overnight. To avoid the apoptotic death reported in serum-deprived Schwann cells (Delaney *et al.* 1999) cultures were switched to serum-free medium without an intermediate rinse step. This method allows for the maintenance of primary Schwann cell cultures for up to 18 h. Cells were loaded with 3 µM fura-2 acetoxymethyl ester (Fura-2AM; Molecular Probes, Eugene, OR, USA) for 20 min at 37°C in HBSS [in mM: 137.0 NaCl, 5.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 0.44 KH₂PO₄, 0.34 Na₂HPO₄(7H₂O), 20.0 Na⁺ HEPES, 1.0 NaHCO₃ and 5.0 glucose, pH 7.4], rinsed twice and imaged in the same medium at room temperature using the Zeiss AttoFluor RatioVision system (Rockville, MD, USA). Coverslips were mounted in an AttoFluor chamber with a maximum holding volume of 1 mL. The chamber was filled with 1 mL of warm HBSS and mounted onto the microscope stage of a Zeiss Axiovert 135 inverted microscope equipped for digital fluorescence microscopy. A computer image of the cells was captured using an ICCD camera and 25–30 cellular regions of interest were selected to be digitally monitored. Baseline ratios of Fura-2 fluorescence emission at 520 nm were measured during high-frequency alterations of 334 nm (Ca²⁺ bound) and 380 nm (Ca²⁺ free) excitation filters. Following 90 s of baseline measurements, 10 µL of 1 mM U46619 (Biomol, Plymouth Meeting, PA, USA), a stable TXA₂ mimetic, was diluted into the 1 mL holding volume (≈10 µM final concentration) and assayed for a change in Fura-2 ratio for 90 s. In some experiments, TP receptor antagonists were added after baseline measurements were obtained. Thromboxane receptor antagonists BMS180,291, BM13,505 were generous gifts of Dr K. Stegmeier (Boehringer Mannheim GmbH, Germany). The TP receptor antagonist SQ29,548 was obtained from Biomol. Following 90 s of antagonist treatment, U46619 was added and the cells were further monitored for a calcium response. In the absence of a calcium transient, 10 µM ATP (RBI) was added to elicit a

physiologic response prior to termination of the experiment. All experiments were performed in triplicate. Representative experiments are reported for each experiment.

Determination of thromboxane B₂ levels in conditioned medium

Primary rSC and T265 cells were seeded onto 60-mm culture dishes at 80–85% confluency. Cells were rinsed twice with low-glucose DMEM (primary rSC) or high-glucose DMEM (T265 cells) and held in 4 mL of serum-free DMEM medium for 24 h at 37°C in a humidified incubator (5% CO₂/95% air). Conditioned medium was collected and assayed indirectly for the presence of TXA₂ using an ELISA specific for TXB₂ (Assay Designs Inc., Ann Arbor, MI, USA); a stable metabolite of TXA₂. To determine TXA₂ levels in proliferating rSC, primary rSC were seeded onto 60-mm culture dishes as described above and stimulated with 50 ng/mL Neu-differentiation factor (NDFβ; R & D Systems), a potent mitogen for primary rSC, for 24 h. Thromboxane B₂ was not detected in stock media solutions containing 5% FBS.

Determination of intracellular cAMP levels ([cAMP]_i)

Primary rSC and T265 cells were seeded onto 24-well culture plates at a density of 100 000 cells/well in low-glucose DMEM (primary rSC) or high-glucose DMEM (T265 cells) and then serum deprived overnight. All treatments were performed using serum-free medium containing 200 μM 3-isobutyl-1-methylxanthine (Sigma). Solutions containing concentrations of TP receptor agonists spanning four log units (1 nM to 10 μM) were applied for 10 min at 37°C. Specificity of cAMP changes in response to U46619 was determined by pre-incubating cells with varying doses of the TP receptor antagonist BMS180,291 (10 nM to 10 μM) for 5 min followed by 10 μM U46619 for 10 min at 37°C. Following treatment, the media was aspirated completely and the cells were lysed using 200 μL of 0.1 M HCl applied for 1 h at room temperature on a rotary shaker. Lysates were collected in 500 μL centrifuge tubes, centrifuged at 500 g for 10 min, and used immediately. [cAMP]_i were determined in triplicate using the acetylated version of a direct enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's instructions. Data are expressed as fold elevation of cAMP over the basal or non-stimulated state. Mean levels of cAMP content for each condition were determined by averaging values obtained from three separate assays.

Results

Identification of TP receptor protein expression in primary rSC and T265 cells

To determine whether primary rSC and T265 cells expressed TP receptors, we performed immunocytochemical and immunoblot analyses using polyclonal antibodies raised against either the purified TP receptor protein (TxAb) or a decapeptide sequence from the first extracellular loop of the TP receptor (P2Ab; residues 89–98). Both primary rSC and T265 cells demonstrated immunoreactivity for TP receptors (Fig. 1). In T265 cells, TP receptor immunoreactivity was detected predominantly on the cytoplasmic membrane. Similar immunoreactivity was obtained in T265 cells using either TxAb (Fig. 1a) or P2Ab (Fig. 1b). In primary

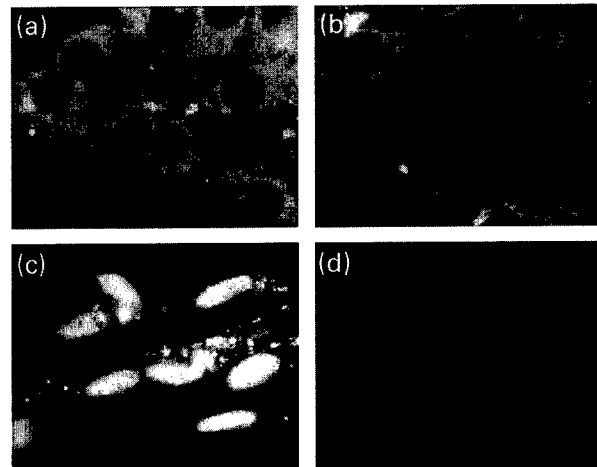


Fig. 1 Immunocytochemical detection of TP receptors. T265 cells were fluorescently labeled using either a polyclonal anti-TP receptor antibody (a, 1 : 50) or a polyclonal anti-peptide antibody (P2Ab; amino acids 89–98) (b, 1 : 50). Incubation of primary rSC with P2Ab (c, 1 : 50) also demonstrated positive immunoreactivity. Incubation of T265 cells with pre-immune IgG from the animal used to create the P2Ab anti-peptide antibody did not produce any detectable immunoreactivity (d). antibody immunoreactivity was visualized using a goat-antirabbit secondary antibody conjugated with FITC (1 : 100).

rSC, immunoreactivity for TP receptors was localized throughout the cellular cytoplasm as well as within the nuclei of the cells (Fig. 1c). Cellular immunoreactivity was not detected using a pre-immune IgG obtained from the animal used to generate P2Ab (Fig. 1d). Similar results were obtained using pre-immune IgG from the animal used to

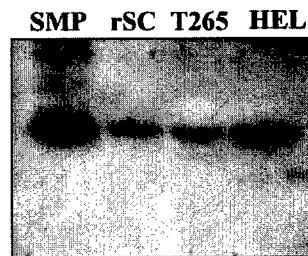


Fig. 2 Identification of TP receptor in protein lysates from primary rSC and T265 cells. Twenty micrograms of protein lysate was resolved electrophoretically on a 4–20% polyacrylamide gel and transferred to PVDF for immunoblot analysis. Incubation with TXA₂ receptor antibody (1 : 1000 dilution) revealed immunoreactivity for a 55-kDa protein in lysates from primary rSC (lane 2) and T265 cells (lane 3) that corresponded to a 55-kDa protein found in lysates obtained from human platelet membranes (SMP, 10 μg; lane 1) and human erythroleukemia cell line (HEL, 20 μg; lane 4). Incubation of PVDF membranes with pre-immune immunoglobulins from the same animal used to produce the anti-peptide antibody did not produce significant immunoreactivity in any of the lysates tested (data not shown).

generate TxA₂ (data not shown). To confirm that primary rSC and the T265 cells expressed TP receptor protein, we separated membrane protein extracts using SDS-PAGE and analyzed the immunoblots for the presence of TP receptor using the P2Ab. A single, prominent immunoreactive band was present at 55 kDa in both primary rSC and T265 cell extracts (Fig. 2, lanes 2 and 3). As a positive control for TP receptor immunoreactivity, a protein extract of human platelet membranes (Fig. 2, lane 1, SMP) and an extract from a human erythroleukemia cell line (Fig. 2, lane 4, HEL) were electrophoresed in parallel with Schwann cell lysates. The immunoreactive band in both primary rSC and T265 lysates co-migrated with immunoreactivity found in both SMP and HEL extracts (55 kDa). Thromboxane receptor immunoreactivity was not detected in lysates incubated with a pre-immune IgG obtained from the animal used to generate the TP receptor antibody (data not shown). Collectively, these results demonstrate that both primary rSC and T265 cells express TP receptor protein.

Radioligand binding analysis of TXA₂ binding sites in primary rSC and T265 cells

To determine whether the protein visualized by immunocytochemistry and immunoblot analysis represented functional ligand binding sites, saturation binding of the specific TP receptor antagonist [³H]SQ29,548 to both primary rSC and T265 cells was performed using adherent cell cultures (Fig. 3a,b). These experiments revealed a single class of high-affinity binding sites for the ligand in both rSC and T265 cells. Primary rSC binding sites had a dissociation constant (K_D) of 3.58 ± 3.2 nM and a maximum binding (B_{max}) of 2719.63 ± 830.9 fmol/mg protein. T265 cell-binding sites were found to have a K_D of 9.69 ± 7.46 nM and a B_{max} of 196.24 ± 72.08 fmol/mg protein. The binding kinetics of both rSC and T265 cells are comparable with those obtained for the thromboxane receptor in human oligodendroglia cells ($K_D = 4.0$ nM; Blackman *et al.* 1998), 1321N1 human astrocytoma cells ($K_D = 10.9$ nM; Nakahata *et al.* 1992) and human platelets ($K_D = 7.3$ nM; Hedberg *et al.* 1988).

The effect of TP receptor stimulation on intracellular calcium levels

Given that both primary rSC and T265 cells demonstrated immunoreactivity for the TP receptor as well as binding of a receptor-specific antagonist, we tested the functionality of these receptors by digital fluorescence calcium imaging using the calcium-sensitive dye Fura-2. Addition of the TXA₂ mimetic U46619 (10 μ M) produced a robust calcium transient in the T265 cell line (Fig. 4a). In contrast, primary rSC did not respond to 10 μ M U46619 with an increase in intracellular calcium (Fig. 4a). Primary Schwann cell cultures were responsive to stimulation with 10 μ M ATP. Schwann cells isolated from Lewis rats have been shown to produce TXA₂ (Constable *et al.* 1994). Thus, we hypothesized

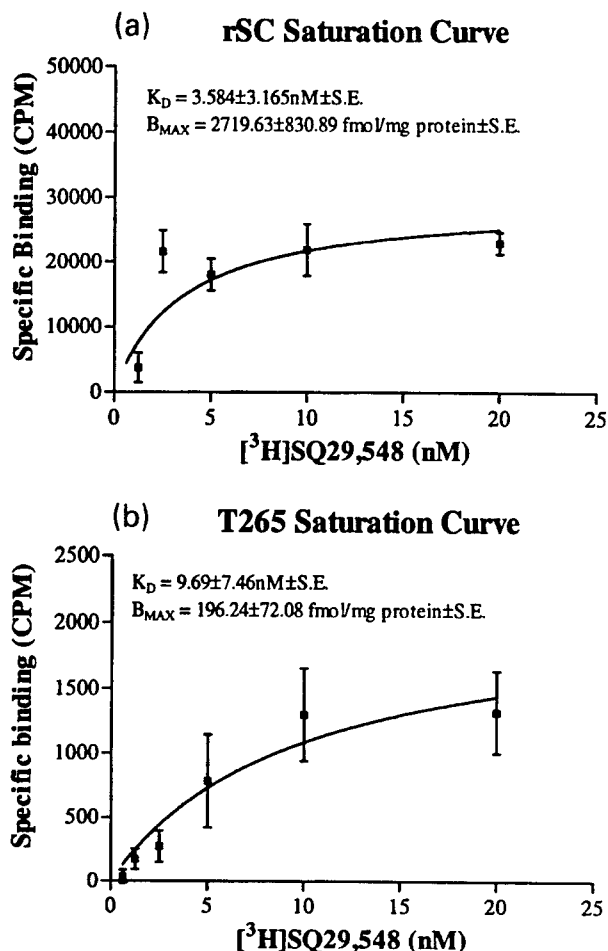


Fig. 3 Radioligand binding analysis of primary rSC and T265 cells. [³H]SQ29,548 binding was measured in the absence (total binding) or presence (non-specific binding) of 20 μ M unlabeled SQ29,548 for 30 min. Saturation curves demonstrate the difference between total and non-specific binding (specific binding). (a) Saturation curve for [³H]SQ29,548 binding to primary rat Schwann cells. (b) Saturation curve for [³H]SQ29,548 binding to T265 cells. Data are the average of two (rSC) or four (T265) experiments performed using triplicate determinations of total and non-specific binding for each experiment.

that the lack of physiological calcium response in primary rSC may have been the result of TP receptor desensitization due to endogenous TXA₂ production and release. To test this possibility, we incubated primary rSC with indomethacin (100 nM) for 24 h to inhibit any endogenous TXA₂ production. Primary rSC treated with indomethacin did not respond to U46619 stimulation with an increase in intracellular calcium (data not shown).

Determination of TXA₂ release by primary rSC and T265 cells

To determine the level of production and secretion of TXA₂ by the cells used in this study, we measured TXA₂ levels in conditioned medium from primary rSC and T265 cells after

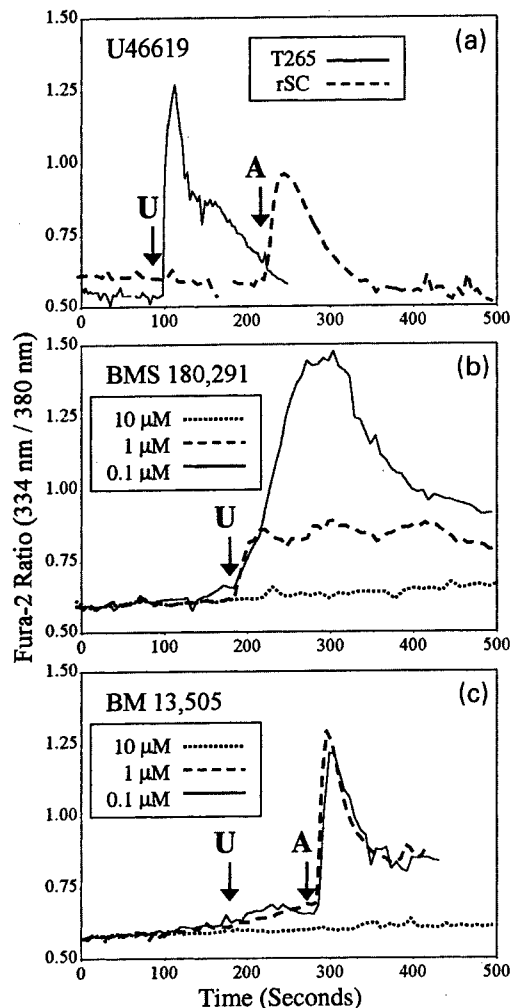


Fig. 4 Analysis of intracellular calcium levels following TP receptor stimulation. (a) Stimulation of T265 cells and primary rSC with 10 μ M U46619 (arrow, U) elicited a robust calcium transient in T265 cells but not in primary rSC. Primary rSC were responsive to 10 μ M ATP (arrow, A). (b) Prior treatment of T265 cells with the specific TP receptor antagonist BMS180,291 inhibited calcium transients in response to 10 μ M U46619 (arrow, U) in a dose-dependent fashion. Complete response inhibition was observed following treatment with 10 μ M BMS180,291 and a gradual recovery of calcium transients was observed with decreasing doses of the antagonist. In comparison to antagonist-naïve T265 cells (a, solid tracing) antagonist-treated cells responded to U46619 with less synchrony resulting in a broadened calcium tracing (b, solid tracing) or several peaks (b, dashed tracing) indicative of variable response latencies. (c) Pre-treatment of T265 cells with the specific TP receptor antagonist BM13,505 inhibited calcium transients in response to 10 μ M U46619 (arrow, U). Addition of 10 μ M ATP (solid and dashed tracings, arrow, A) was used to obtain a physiologic response prior to terminating the experiment.

24 h. Using an ELISA specific for TXB₂, a stable metabolite of TXA₂, we found that both quiescent and actively proliferating rSC (50 ng/mL NDF β for 24 h) did not release any detectable TXB₂ into the medium. In contrast,

conditioned medium from T265 cells contained 1.1 ng/mL TXB₂. The concentration of TXB₂ in 1 mL of conditioned medium was calculated to be 2.7 pM, a level of production several orders of magnitude lower than the agonist concentrations utilized in our experiments.

Effect of TP receptor antagonists on U46619-stimulated calcium transients in T265 cells

To determine the specificity of U46619-mediated calcium elevation in T265 cells, we treated T265 cells with various doses of the TP receptor antagonists, BMS180,291 (Fig. 4b) and BM13,505 (Fig. 4c). For each individual experiment, a baseline ratio of Fura-2 fluorescence was obtained for 90 s followed by TP receptor antagonist treatment for 90 s. Following 180 s of ratio measurement, 10 μ M U46619 was added to the cells (arrow, U). In the absence of a response to U46619, ATP (10 μ M, arrow, A) was added in several experiments. A dose-dependent inhibition of U46619-stimulated calcium elevation was observed using BMS180,291. For the highest dose of BMS180,291 tested (10 μ M), complete inhibition of calcium elevation was observed. Compared with antagonist-naïve T265 cells (Fig. 4a), T265 cells treated with 1 μ M BMS180,291 exhibited a moderate response that was prolonged in duration. Analysis of individual cell tracings reveals a variable response onset to U46619 stimulation (data not shown). A similar distribution of cellular responses across time was present for the lowest dose of BMS180,291 tested (0.1 μ M), producing a broadened calcium transient (data not shown). However, in the case of 0.1 μ M BMS180,291, no significant inhibition of U46619-stimulated calcium elevation was observed. In contrast to BMS180,291, the TP receptor antagonist BM13,505 inhibited U46619-stimulated calcium elevation for each dose tested (Fig. 4c). T265 cells remained responsive to 10 μ M ATP (second arrow, A). Inhibition of calcium elevation in T265 cells following U46619 treatment was also observed using 10 μ M of the TP antagonist SQ29,548 (data not shown).

Effect of TP receptor agonists on [cAMP]_i

In an effort to identify a functional response for TP receptors in primary rSC we measured [cAMP]_i following treatment with U46619. Both primary rSC and the T265 cell line responded to U46619 treatment by an increase in [cAMP]_i following 10 min of stimulation (Fig. 5a). Elevations in [cAMP]_i following U46619 stimulation were dose dependent in both primary rSC (gray columns) and the T265 cells (black columns). Compared with non-stimulated conditions, the T265 cell line exhibited a 12- to 14-fold elevation in [cAMP]_i at the maximum concentrations of U46619 tested (1 and 10 μ M). Similarly, in primary rSC, [cAMP]_i was elevated between 14- (1 μ M) and 22-fold (10 μ M) over basal conditions following stimulation with U46619. In both rSC and T265 cells, elevations in [cAMP]_i in response to

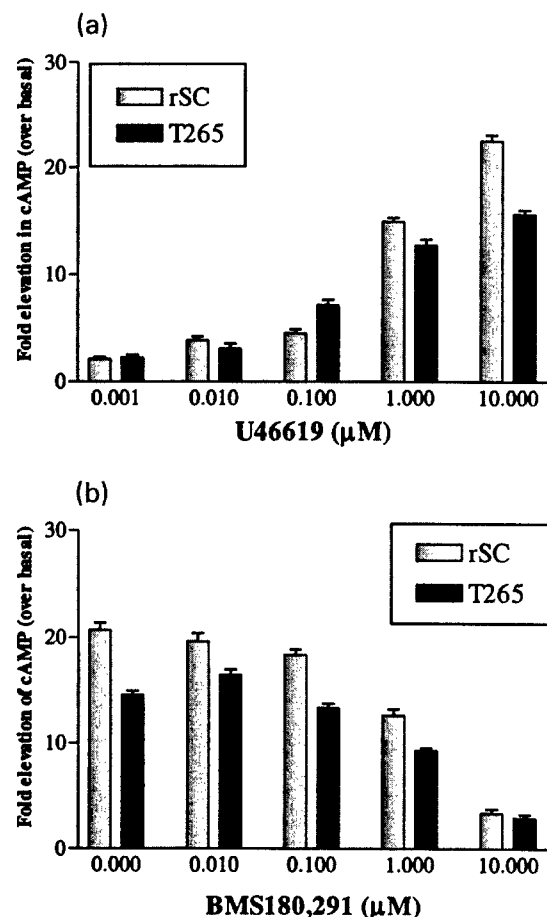


Fig. 5 Dose-dependent elevation of $[\text{cAMP}]_i$ following TP receptor stimulation. T265 cells and primary rSC treated with U46619 exhibited a dose dependent elevation in $[\text{cAMP}]_i$ following 10 min of receptor stimulation in the presence of $200 \mu\text{M}$ 3-isobutyl-1-methylxanthine (a). BMS180,298 inhibited cAMP accumulation in response to $10 \mu\text{M}$ U46619 in a dose-dependent manner in both cell types (b). For both cell types, maximum $[\text{cAMP}]_i$ were obtained using either 10 or $1 \mu\text{M}$ doses of U46619. Error bars represent the SEM of three separate experiments. Basal levels of cAMP/100 000 cells were 1.4 and 2.7 pM for primary rSC and T265, respectively.

U46619 stimulation were inhibited by the TP receptor antagonist BMS180,291 in a dose-dependent fashion (Fig. 5b). Basal levels of cAMP/100 000 cells were 1.4 and 2.7 pM for primary rSC and T265, respectively.

Phosphorylation of CREB on Ser133 following TP receptor stimulation

To determine whether the intracellular levels of calcium and cAMP in rSC and T265 cells were sufficient to stimulate downstream signaling events to a significant degree, we performed immunoblot analyses of CREB phosphorylation following TP receptor stimulation. The addition of $10 \mu\text{M}$ U46619 produced an elevation in CREB phosphorylation on Ser133 in both primary rSC and the T265 cell line (Fig. 6).

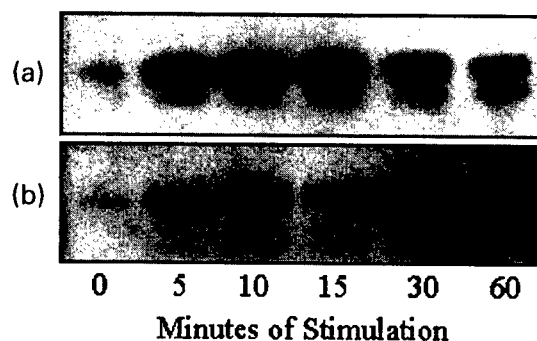


Fig. 6 Time course of CREB phosphorylation on Ser133 following TP receptor stimulation with $10 \mu\text{M}$ U46619. T265 cells and primary rSC were stimulated with $10 \mu\text{M}$ U46619 for 0, 5, 10, 15, 30 or 60 min and then lysed in preparation for immunoblot analyses of CREB phosphorylation. Immunoblot analyses demonstrate that CREB was phosphorylated within 5 min of stimulation in both T265 cells (a) and primary rSC (b).

Cyclic AMP response-element binding protein phosphorylation was strongly elevated in T265 cells following 5 min of stimulation (Fig. 6a). Cyclic AMP response-element binding protein phosphorylation was also detected in primary rSC following 5 min of treatment with maximal levels achieved following 10 min of stimulation (Fig. 6b). In both cell types, the level of CREB phosphorylation remained elevated for at least 45 min with a decrease towards basal levels after 60 min of stimulation. In T265 cells, a second immunoreactive band was detected below that corresponding to phosphorylated CREB at later time points (15–60 min). The identity of this second immunoreactive band has been shown to correspond to ATF-1, a molecule related to CREB family of transcription factors (Ginty *et al.* 1993). Interestingly, CREB was phosphorylated to a higher degree in T265 cells than in primary rSC. Using identical protein concentrations and immunoblotting conditions, immunoblots of phosphorylated CREB using T265 lysates routinely required 10-fold less exposure (30 s) than primary rSC lysates (5 min) for significant signal to develop. To further characterize the extent of CREB phosphorylation following U46619 stimulation, we performed immunocytochemistry using the T265 cell line. Compared with background levels of immunoreactivity (Fig. 7a), unstimulated T265 cells demonstrated low levels of basal CREB phosphorylation in the nuclear cytoplasm (Fig. 7b). Stimulation with $10 \mu\text{M}$ U46619 for 15 min produced a significant increase in nuclear immunoreactivity in these cells (Fig. 7c). Significant differences in nuclear staining intensity for phosphorylated CREB were not observed between non-stimulated primary rSC and primary rSC treated with U46619 (data not shown), consistent with the observation that immunoblot analyses of phosphorylated CREB in primary rSC required 10-fold longer exposures than in T265 cells.

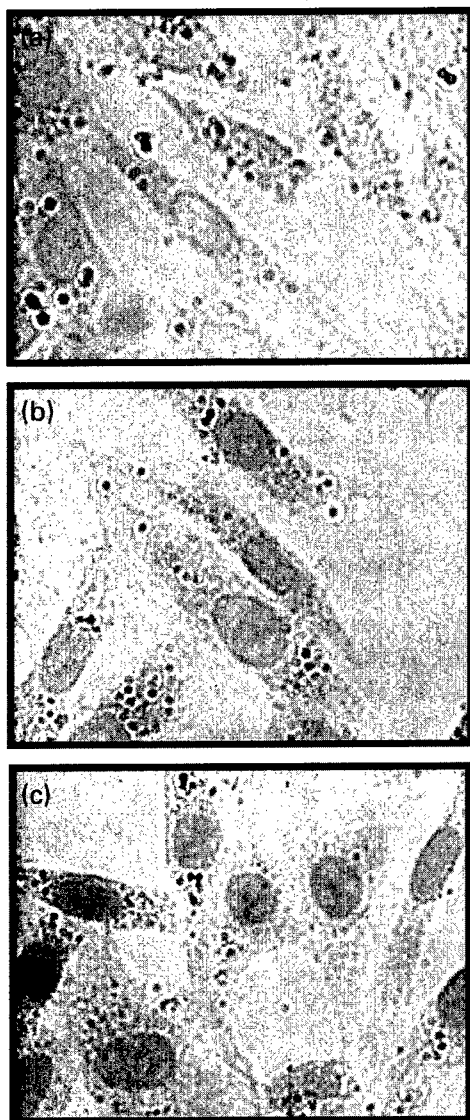


Fig. 7 Stimulation of CREB phosphorylation on Ser133 following TP receptor stimulation. Compared with labeling with secondary antibody alone (a), unstimulated T265 cells demonstrated low, basal levels of nuclear immunoreactivity for phosphorylated CREB (b, arrowheads). T265 cells stimulated with 10 μ M U46619 for 15 min demonstrated increased nuclear immunoreactivity for antibodies raised against CREB phosphorylated on Ser133 (c, arrowheads).

Discussion

In this report, we demonstrate that both primary rSC and T265 cells express TP receptors using immunochemical and functional approaches. Immunoblot analyses reveal the presence of a 55-kDa protein that is reactive with an antibody raised against both a TP receptor decapeptide sequence (P2Ab) and purified human platelet TP receptor (TxAb) (Fig. 2). Immunocytochemical analysis using both of these antibodies demonstrated specific immunoreactivity

(Fig. 1). Of note, although both primary rSC and T265 cells were labeled on the cytoplasmic membrane, only primary rSC demonstrated staining of both the nuclear and cytoplasmic membranes. Recent reports have identified functional EP2, EP3 and EP4 prostaglandin receptors within the nuclear envelope of HEK 293 and porcine cerebral microvascular endothelial cells (Bhattacharya *et al.* 1998, 1999). These findings, along with the data presented here, raise the interesting possibility that TP receptors may also be present within the nuclear envelope in Schwann cells and in other cell types.

Experiments were performed to evaluate the ability of intact rSC and T265 cells to bind a TP receptor ligand ($[^3\text{H}]\text{SQ29,548}$). The results demonstrated that rSC cells express 2719 fmol of receptor per mg protein with a K_D value of ≈ 3.6 nM. T265 cells, however, exhibit ≈ 196 fmol of receptor per mg protein with a K_D value of ≈ 9.7 nM. These data reveal a 13-fold difference in receptor density between primary rat and tumor-derived human Schwann cells. It is possible that the lower receptor density seen in T265 cells is due to altered expression of native protein secondary to transformation. Alternatively, it is possible that the T265 cell behaves as a less differentiated form of Schwann cell and that TP receptor expression varies with cell differentiation. It should be noted, however, that the K_D for both Schwann and T265 cells reported here are comparable with values observed in human platelets as well as in astrocytoma and oligodendroglioma cell lines (Nakahata *et al.* 1992; Blackman *et al.* 1998).

Functional characterization of TP receptors revealed that distinct intracellular signaling mechanisms were activated following receptor activation in these two cell types (Figs 4 and 5). Receptor stimulation with the stable TXA₂ mimetic U46619 produced a robust calcium transient in T265 cells that could be inhibited by prior treatment with several different TP receptor antagonists. In contrast to T265 cells, stimulation with U46619 did not elevate intracellular calcium in primary rSC. Interestingly, TP receptor stimulation also produced a specific, dose-dependent elevation in $[\text{cAMP}]_i$ in T265 cells as well as in primary rSC. Furthermore, a time-dependent elevation in the level of CREB phosphorylation on Ser133 was also detected in both T265 cells and primary rSC. Cyclic AMP response-element binding protein phosphorylation can be stimulated by diverse intracellular mechanisms that include both calcium- and cAMP-dependent signaling pathways (Montminy 1997; Shaywitz and Greenberg 1999). Because U46619 stimulated an elevation in intracellular calcium and $[\text{cAMP}]_i$ in T265 cells, it is possible that one or both of these signaling pathways contributed to the subsequent phosphorylation of CREB. However, because U46619 stimulation did not affect intracellular calcium levels in primary rSC, it appears that CREB was phosphorylated mainly by a cAMP-dependent mechanism in these cells. Compared with primary rSC,

CREB was phosphorylated more rapidly and to a higher degree in T265 cells (Fig. 6a), suggesting that calcium elevation following U46619 stimulation may contribute significantly to CREB phosphorylation.

Physiologic factors which stimulate cAMP in Schwann cells

It is well established that elevations in $[cAMP]_i$ in Schwann cells have profound effects on Schwann cell physiology (Sobue and Pleasure 1984). The plant diterpene forskolin, cholera toxin and non-hydrolyzable, cell-permeant analogs of cAMP are the most common pharmacological compounds used to elevate $[cAMP]_i$ in primary rSC (Yamada *et al.* 1995). However, despite the frequent application of these pharmacologic agents in Schwann cell biology, the absolute $[cAMP]_i$ that are attained by these non-physiologic means have yet to be determined and few physiologic factors that stimulate cAMP accumulation in primary rSC have been identified. Schwann cells have been shown to respond to both β -adrenergic ligands (Yasuda *et al.* 1988) and calcitonin gene-related peptide (Cheng *et al.* 1995) by an elevation in $[cAMP]_i$. Here, we identified U46619 as novel physiologic analog that is capable of stimulating intracellular cAMP accumulation and CREB phosphorylation in primary rSC and T265 cells. Activation of TP receptors has been shown to functionally couple to a wide variety of G-proteins including G_q (Knezevic *et al.* 1993; Allan *et al.* 1996) and $G_{12/13}$ (Offermanns *et al.* 1994; Allan *et al.* 1996; Djellas *et al.* 1999). In addition, TP receptors have been shown to modulate the activity of adenylyl cyclase, although the exact mechanism by which this occurs is unclear. Although both $TP\alpha$ and $TP\beta$ receptor isoforms activate phospholipase C to a similar extent, activation of $TP\alpha$ in transfected cells has been shown to elevate $[cAMP]_i$, whereas activation of $TP\beta$ in transfected cells decreases adenylyl cyclase activity (Hirata *et al.* 1996; Walsh *et al.* 1998; Cracowski *et al.* 2000). In primary rSC and T265 cells, U46619 stimulated a dose-dependent elevation of $[cAMP]_i$, suggesting that these cells may both express predominantly $TP\alpha$. The levels of $TP\alpha$ and $TP\beta$ and the contribution of these receptors to intracellular cAMP accumulation in primary rSC and T265 cells remains to be determined as the antibodies used in this study were not devised to distinguish between the two TP receptor isoforms.

Schwann cells as a potential source of eicosanoids within peripheral nerve

In our studies, significant levels of TXB_2 , a stable metabolite of TXA_2 , were not detected in medium conditioned by primary rSC. In contrast, TXB_2 was detected in medium conditioned by T265 cells. Schwann cells isolated from Lewis rats have been shown to produce significant amounts of TXA_2 under both basal conditions and following an experimental elevation in intracellular calcium levels

(Constable *et al.* 1994). The finding that Schwann cells isolated from Lewis rats have significantly elevated levels of TXB_2 may be species specific, suggesting that the rats used in our study may differ in some respects. Alternatively, the discrepancy may be due to the detection level of the competitive ELISA assay used in our experiments compared with the scintillation proximity assay used by Constable *et al.* (1994), which has an order of magnitude greater sensitivity.

Potential role of TP receptors in diseases affecting peripheral nerve

The identification of functional TP receptors in Schwann cells suggests that the normal or abnormal release of TP within peripheral nerve may modulate Schwann cell physiology under either normal or pathological conditions, respectively. Following peripheral nerve injury, blood-derived macrophages invade the site of injury and then differentiate into activated macrophages (Beuche and Friede 1986; Langley and Pearce 1998). Macrophages participate in the removal of degenerating axonal and myelin debris and the establishment of a local environment that is conducive for axonal regeneration (Heumann *et al.* 1987; Griffin *et al.* 1992; Avellino *et al.* 1995; Dailey *et al.* 1998). Macrophages have been shown to digest and release protein fragments of myelin basic protein from myelin phagocytized at the injury site (Baichwal *et al.* 1988). These myelin basic protein fragments have been shown to be mitogenic in the presence of experimentally elevated cAMP levels in Schwann cells (Tzeng *et al.* 1995). In addition to the production and secretion of cytokines and free radicals, macrophages have long been known to be a major source of eicosanoids including TXA_2 . Our findings suggest that TXA_2 may be a physiologic factor that could operate in conjunction with myelin basic protein fragments to stimulate Schwann cell proliferation following peripheral nerve injury. The discovery of TP receptors in Schwann cells also raises the interesting possibility that these cells may express additional eicosanoid receptors (e.g. EP and IP) that may also be coupled to changes in $[cAMP]_i$.

In sum, this study is the first report of functional TXA_2 receptors in Schwann cells. The receptor was found to have an electrophoretic mobility, immunoreactivity and radioligand-binding kinetics similar to those of platelet and oligodendrogloma TXA_2 receptors. Activation of Schwann cell TP receptors stimulates elevations in $[cAMP]_i$ and the phosphorylation of CREB. Activation of TP receptors in a Schwann cell tumor line leads to elevations in both $[cAMP]_i$ and intracellular calcium. The results presented here indicate that activation of Schwann cell TP receptors leads to the mobilization of cAMP and, in a transformed cell line, both calcium and cAMP, which contributes in part to the translocation of CREB to the cell nucleus. Taken together, these findings suggest a novel pathway for by which

Schwann cells could respond to mediators released during inflammation within the PNS.

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Neurofibrosarcoma-Derived Schwann Cells Overexpress Platelet-Derived Growth Factor (PDGF) Receptors and Are Induced to Proliferate By PDGF BB

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Neurofibromatosis type 1 (NF1) is characterized by the formation of neurofibromas, benign tumors of the peripheral nerve consisting essentially of Schwann cells, which can sometimes turn malignant to form neurofibrosarcomas. The mechanism of progression toward a malignant phenotype remains largely unknown. In this report, we show that platelet-derived growth factor (PDGF) BB, and to a lesser extent fibroblast growth factor 2, are mitogenic for two neurofibrosarcoma-derived Schwann cell lines, but not for a Schwann cell line derived from a schwannoma (from a non-NF1 patient) or for transformed rat Schwann cells. Levels of expression of both PDGF receptor α and β are significantly increased in the two neurofibrosarcoma-derived cell lines compared to the non-NF1 Schwann cell lines. The level of tyrosyl-phosphorylated PDGF receptor β is strongly increased upon stimulation by PDGF BB. In comparison, only modest levels of tyrosyl-phosphorylated PDGF receptor α are observed, upon stimulation by PDGF AA or PDGF BB. Accordingly, PDGF AA is only a weak mitogen for the neurofibrosarcoma-derived cells by comparison to PDGF BB. These results indicate that the mitogenic effect of PDGF BB for the neurofibrosarcoma-derived Schwann cell lines is primarily transduced by PDGF receptor β . Neu differentiation factor β , a potent mitogen for normal Schwann cells, was unable to stimulate proliferation of the transformed Schwann cell lines, due to a dramatic down-regulation of the erbB3 receptor. Therefore, aberrant expression of growth factor receptors by Schwann cells, such as the PDGF receptors, could represent an important step in the process leading to Schwann cell hyperplasia in NF1. *J. Cell. Physiol.* 177:334–342, 1998. © 1998 Wiley-Liss, Inc.

Neurofibromatosis type 1 (NF1) is a common inherited disease affecting one in 3,000 individuals, which manifests with various symptoms including neurofibromas, café-au-lait spots, axillary freckling, bone dysplasia, and learning disabilities (Riccardi et al., 1991). Neurofibromas are benign tumors of the peripheral nerve sheath, composed primarily of Schwann cells, but also fibroblasts, perineurial cells, and mast cells. NF1 patients are at higher risk of developing malignant peripheral nerve sheath tumors, also called neurofibrosarcomas, which may arise from neurofibromas. The defective gene in NF1, identified by positional cloning (Cawthon et al., 1990; Viskoshil et al., 1990; Wallace et al., 1990), codes for a protein called neurofibromin, which shares sequence homology with GTPase-activating proteins (Buchberg et al., 1990; Xu et al., 1990). It was predicted that the loss of neurofibromin would result in increased Ras activity that could lead to Schwann cell hyperplasia (Basu et al., 1992; DeClue et al., 1992). Recent studies confirmed that Ras-GTP levels are elevated in neurofibrosarcomas from NF1 patients (Guha et al., 1996) and in Schwann cells derived from *Nf1* mutant mouse (Kim et al., 1995). Neverthe-

less the actual mechanisms leading to the formation of neurofibromas and neurofibrosarcomas remains unclear. Both epigenetic events and multiple genetic changes have been proposed to contribute to the formation of the peripheral nerve sheath tumors in NF1 (Rosenbaum et al., 1997).

Overexpression of growth factors and/or their receptors is believed to play an important role in cellular transformation. Neurofibromas, which fail to grow when implanted under the skin of nude mice, do grow when implanted into the peripheral nerve, implicating

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nerve growth factors in tumor growth (Lee et al., 1992). However, the growth factors and growth factor receptors promoting neurofibromas and/or neurofibrosarcomas growth remain unknown. We have shown that, in contrast to normal Schwann cells or non-NF1 transformed Schwann cells, neurofibrosarcoma-derived Schwann cell lines expressed high levels of Kit, the tyrosine kinase receptor for stem cell factor (SCF) (Ryan et al., 1994; Badache et al., 1998). Moreover, SCF was able to stimulate the proliferation of neurofibrosarcoma-derived lines. Since the effect of SCF on neurofibrosarcoma-derived Schwann cells was only moderate, we investigated the possibility that, in a manner similar to what has been described in the hematopoietic system (McNiece et al., 1991), SCF has a modest mitogenic effect on its own but may act synergistically with other factors to induce cell proliferation. Several potential co-factors for SCF were tested, including neurodifferentiation factor β (NDF β), a member of a family of growth factors called neuregulins, which are potent mitogens for both human and rat Schwann cells (Marchionni et al., 1992; Levi et al., 1995); platelet-derived growth factor BB (PDGF BB) and fibroblast growth factor 2 (FGF2), well-known mitogens for rat Schwann cells (Davis and Stroobant, 1990); and forskolin, an agent which increases the levels of intracellular cAMP, known to potentiate the mitogenicity of FGF2, PDGF BB, and NDF β , by inducing increased expression of their receptors (Weinmaster and Lemke, 1990; Cohen et al., 1990).

SCF had no synergistic effect on cell proliferation when combined with either NDF β , PDGF BB, FGF2, or forskolin. However, PDGF BB and to a lesser extent FGF2 appeared to be strongly mitogenic on their own. NDF β , the most potent mitogen for normal Schwann cells, had no effect on neurofibrosarcoma-derived Schwann cell proliferation. Further studies revealed drastic changes in the pattern of growth factor receptors expressed by the neurofibrosarcoma-derived Schwann cells. Our results reveal that growth factors, PDGF BB in particular, may play a significant role in the aberrant growth of Schwann cells that leads to tumor formation in NF1.

MATERIAL AND METHODS

Cell lines and growth factors

The ST88-14 cell line (obtained from Jonathan Fletcher, Brigham and Women Hospital, Boston, MA) and the T265-2c cell line (developed by Karen Klein in our laboratory) were derived from malignant peripheral nerve tumors resected from patients with NF1. The STS-26T was derived from an isolated grade III malignant Schwannoma in an individual without NF1 and was obtained from William Dahlberg (Harvard School of Public Health, Boston, MA). The RSC-96 line is a spontaneously transformed rat Schwann cell line derived from long term culture of rat primary Schwann cells. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT). All the cell lines express the Schwann cell markers S100, CNPase, and laminin, as seen by immunocytochemistry and/or immunoblotting. Recombinant human PDGF AA, PDGF BB, FGF2, and SCF were obtained from R&D Systems (Minneapolis, MN). Recombinant NDF β 1 (amino-acids

14-241) was a gift from Amgen (Thousand Oaks, CA). Forskolin was from Calbiochem (La Jolla, CA).

Proliferation assay

To measure cell proliferation, cells were replated in 96-well clusters at 5,000 cells/well. Cells were allowed to adhere to the substratum in DMEM containing 10% FCS and then switched to serum-free DMEM, containing the growth factors at the doses indicated in the text. Media and growth factors were replenished every 3 days. Cell number was evaluated by using the colorimetric MTT assay (Boehringer-Mannheim, Indianapolis, IN) following the manufacturer's protocol. Absorbance was measured at 595 nm and was shown to correlate with viable cell number under the conditions used in this study.

Western blotting and immunoprecipitation

Cells were lysed in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, and SDS, in phosphate buffered saline, PBS, pH 7.2) containing 1 mM sodium orthovanadate and a cocktail of protease inhibitors. Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA). For immunoprecipitation experiments, cells incubated in serum-free medium in the presence of 50 ng/ml PDGF AA or PDGF BB for 15 min were lysed in RIPA buffer. Equivalent amounts of protein were incubated at 4°C for 2 h in the presence of a preformed complex of Protein A/G-Agarose (Santa-Cruz Biotechnology, Santa Cruz, CA) and an antibody specific for PDGF receptor α or β (Oncogene Product, Cambridge, MA). After several washes, the immunoprecipitated proteins were separated by electrophoresis in a 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (Dupont NEN, Boston, MA). After blocking with a 5% non-fat dry milk solution, the PVDF membrane was incubated for 2 h in the presence of the primary antibody (anti-PDGF receptor α , anti-PDGF receptor β , anti-erbB2, anti-erbB3 from Santa Cruz Biotechnology, anti-Kit from Oncogene Product, or anti-phosphotyrosine from Transduction laboratories, Lexington, KY). After several washes in PBS containing 1% Tween-20, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and the immunoreactivity detected by enhanced chemiluminescence (Dupont-NEN, Boston, MA).

RESULTS

Effect of growth factors on neurofibrosarcoma-derived cell proliferation

The effect of SCF, NDF β , PDGF BB, and FGF2 on the growth of two neurofibrosarcoma-derived Schwann cell lines was evaluated; the ST88-14 cell line and the T265-2c cell line are neurofibromin-deficient Schwann cell lines, derived from neurofibrosarcomas from NF1 patients (Badache et al., 1998). Both cell lines expressed the Schwann cell markers S100, CNPase, and laminin as seen by immunocytochemistry and/or immunoblotting (Ryan et al., 1994; Badache, Klein and DeVries, unpublished observations). NDF β , PDGF BB, and FGF2 are known mitogens for rat Schwann cells (Davis and Stroobant, 1990; Raabe et al., 1997). NDF β

is the only potent mitogen for human Schwann cells (Levi et al., 1995; Lopez and DeVries, unpublished observations). Cells were cultured in serum-free medium in the presence of 20 ng/ml growth factor and cell number was evaluated after 4, 7, and 10 days after treatment. SCF had a moderate effect on ST88-14 cell proliferation, as shown before (Fig. 1A). Surprisingly NDF β mitogenicity for the ST88-14 cell line was only modest and comparable to SCF effect. On the other hand, PDGF BB, and to a lesser extent FGF2, which are not mitogens for human Schwann cells (Li et al., 1996), elicited a strong mitogenic response from the ST88-14 cells. After 7 days in culture, the rate of cell growth was slower, most probably due to cell death due to culturing the cells in the serum-free medium. Similar results were obtained using the T265-2c cell line (Fig. 1B): PDGF BB and FGF2 were strong mitogens for these cells, whereas SCF was a weak mitogen. NDF β did not stimulate proliferation of the T265-2c cells. Similar experiments were performed using Schwann cell lines, not derived from NF1 patients. We used the spontaneously transformed rat cell line RSC-96 and a Schwann cell line derived from a Schwannoma in a non-NF1 patient, called STS-26T. SCF is not a mitogen for the STS-26T cell line (as we have previously shown, Badache et al., 1998), nor for the RSC-96 line (Fig. 1C,D). NDF β , FGF2, and PDGF BB also failed to stimulate significantly the growth of the two non-NF1 Schwann cell lines even after 10 days in culture (Fig. 1C,D).

PDGF BB and FGF2 induced the proliferation of the ST88-14 cell line (data not shown) and the T265-2c cell line in a dose-dependent manner (Fig. 2A,B): both PDGF BB and FGF2 significantly stimulated cell growth at doses as low as 0.1 to 1 ng/ml and had a maximal effect around 50 ng/ml. Higher doses of PDGF BB or FGF2 did not further increase the rate of proliferation. When cultured in the presence of 50 ng/ml PDGF BB or FGF2, over a period of five days, T265-2c cell number was increased approximately two-fold and five-fold by FGF2 and PDGF BB, respectively.

To investigate whether, similarly to what is observed in the hematopoietic system (McNiece et al., 1991), SCF can synergize with other growth factors to promote Schwann cell proliferation, we have evaluated the effect of SCF in combination with PDGF BB, FGF2, or NDF β on neurofibrosarcoma-derived Schwann cell proliferation. SCF did not significantly influence the mitogenicity of PDGF BB, FGF2, or NDF β for the T265-2c cells (Fig. 3A) or the ST88-14 cell line (not shown) over the period of time investigated. Combination of SCF with NDF β , PDGF BB or FGF2 did not induce a mitogenic response of the schwannoma-derived cell line, STS26T (Fig. 3B) or the rat cell line RSC-96 (data not shown).

Agents that increase intracellular cAMP, like forskolin, strongly potentiate the mitogenicity of most Schwann cell growth factors including FGF2, PDGF BB, and neuregulins (Davis and Stroobant, 1990; Goodearl et al., 1993; Levi et al., 1995). Therefore, growth factor induced-proliferation of the neurofibrosarcoma-Schwann cells was also evaluated in the presence of 5 μ M forskolin (Fig. 3A). Surprisingly, forskolin had no synergistic effect on the mitogenicity of SCF, NDF β , FGF2, or PDGF BB or combinations of SCF with

the other growth factors. Proliferation of the neurofibrosarcoma-derived Schwann cell lines in the presence or absence of growth factors was significantly decreased by forskolin over 7 days. Over longer period of times forskolin appeared cytotoxic to the cells. Interestingly, PDGF BB and to a lesser extent FGF2 were able to protect the cells from forskolin-induced cell death. Similarly to what we observed for the neurofibromin-deficient cell lines, forskolin did not potentiate the effect of the growth factors on the RSC-96 and STS-26T cells (Fig. 3B). However, forskolin appeared less cytotoxic to the latter. It is noteworthy that the RSC-96 and STS-26T were able to maintain a sustained proliferation rate in the absence of serum, over the period of time investigated (not shown).

Thus, PDGF BB and to a lesser extent FGF2 are strong mitogens for neurofibrosarcoma-derived Schwann cell lines. By comparison, NDF β and SCF are only weak mitogens. Forskolin did not potentiate the mitogenicity of any of the growth factors studied for the neurofibrosarcoma-derived Schwann cells.

Growth factor receptors in the neurofibrosarcoma-derived Schwann cell lines

We then investigated whether the responsiveness of neurofibrosarcoma-derived Schwann cells to PDGF but not to NDF β could be explained by changes in expression of the receptors for these growth factors. We examined expression of the receptors for PDGF (namely PDGF receptor α and PDGF receptor β), for NDF β (erbB2 and erbB3) and SCF (Kit) in the various cell lines, by immunoblotting. PDGF receptor α is expressed at high levels only in the ST88-14 and T265-2c cell lines (Fig. 4A). It is virtually absent in the STS-26T cell line and expressed at low levels in the RSC-96 lines. Similarly, PDGF receptor β was highly expressed only in the two neurofibromin-deficient cell lines (Fig. 4A). PDGF receptor β was expressed at very low levels in the STS-26T cell line. We also confirmed that Kit was highly expressed only in the ST88-14 and T265-2c Schwann cell lines (Fig. 4A), as we have previously described.

ErbB2, the heterodimerization partner of NDF β receptor erbB3, was expressed in all cell lines but the schwannoma-derived STS-26T cell line (Fig. 4B). Contrary to what was observed in primary Schwann cells, erbB3 was not expressed at detectable levels in the tumor-derived Schwann cell lines (Fig. 4B).

Activation of PDGF receptors by PDGF BB in the T265-2c cells

To measure the activation of PDGF receptors activation upon PDGF BB stimulation, T265-2c cells were cultured in the presence of 20 ng/ml PDGF BB for increasing periods of time (from 1 min to 2 h) and the levels of tyrosyl-phosphorylated PDGF receptors analyzed by immunoblotting, by using an anti-phosphotyrosine specific antibody (Fig. 5). Tyrosyl-phosphorylation of the receptor was significantly increased (three- to four-fold over basal levels) less than 1 min after stimulation by PDGF BB and reached a maximum (corresponding to an average ten-fold increase over basal level) after 10–15 min of stimulation. Tyrosyl-phosphorylation was down to basal levels within 2 h. The changes in tyrosyl-phosphorylation were not due to

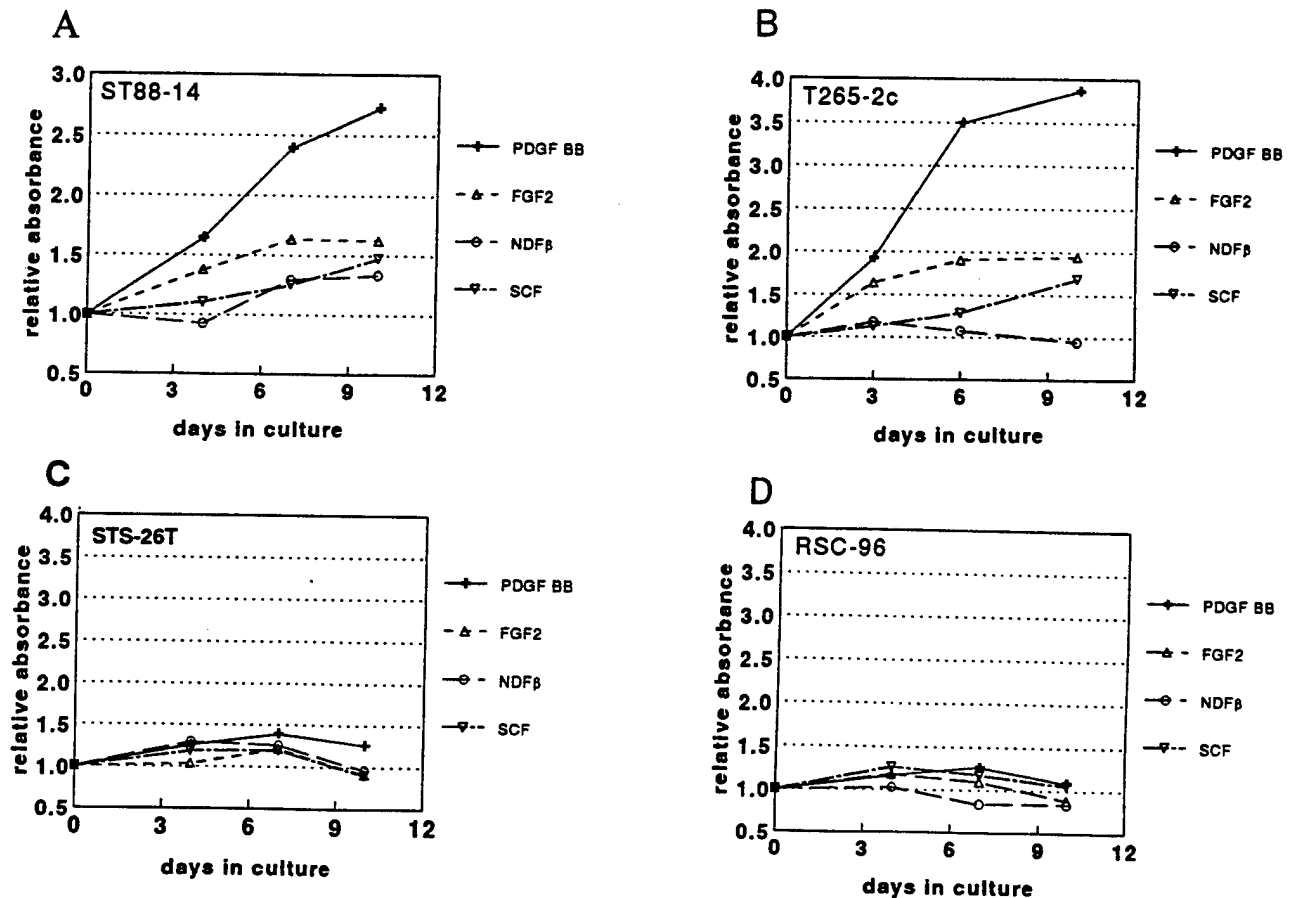


Fig. 1. PDGF BB and FGF2 are mitogenic for neurofibrosarcoma-derived Schwann cells. The neurofibrosarcoma-derived cell lines ST88-14 (A) and T265-2c (B), the schwannoma-derived cell line STS26T (C) and the rat Schwann cell line RSC-96 (D) were cultured in serum-free medium in the presence of 20 ng/ml PDGF BB, FGF2

or SCF or 50 ng/ml NDFβ. Cell number was evaluated over 10 days by the colorimetric MTT assay and expressed relative to untreated cells. Values are the results of four to six replicates from a representative experiment repeated two (C and D) or three times (A and B). Standard deviation did not exceed 10%.

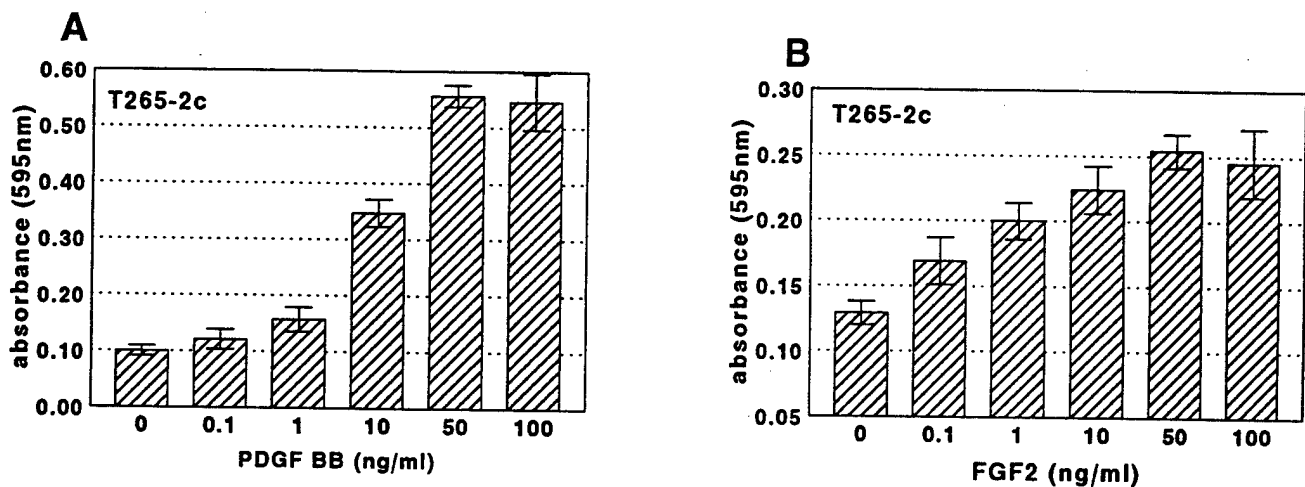


Fig. 2. PDGF BB- and FGF2-induced proliferation of neurofibrosarcoma-derived Schwann cell is dose-dependent. T265-2c cells were cultured in serum-free medium in the presence of increasing amount of PDGF BB (A) or FGF2 (B). Cell number was evaluated by the colorimetric MTT assay after 6 days in culture. Values are expressed as mean \pm standard deviation of six replicates from a representative experiment repeated at least three times.

metric MTT assay after 6 days in culture. Values are expressed as mean \pm standard deviation of six replicates from a representative experiment repeated at least three times.

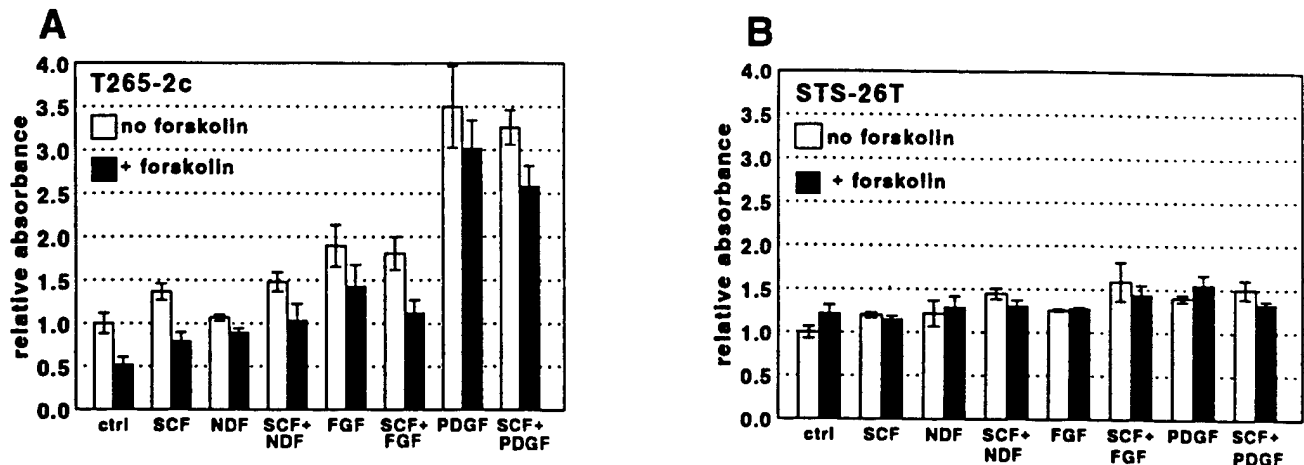


Fig. 3. Effect of NDF β , FGF2, PDGF BB and forskolin on SCF-induced Schwann cell lines proliferation. The neurofibrosarcoma-derived cell line, T265-2c (A) and the schwannoma-derived STS-26T (B) were cultured in serum-free medium for 6 days, in the presence of 20

ng/ml SCF combined with 50 ng/ml NDF β , 20 ng/ml FGF2, or 20 ng/ml PDGF BB in the absence (white bars) or presence (dark bars) of 5 μ M forskolin. Cell number was evaluated as described in Figure 2.

changes in the levels of PDGF receptor expression. Indeed, levels of PDGF receptor β and PDGF receptor α remained unchanged within the first 30 min of the experiment, as seen after reprobing of the membrane with antibodies specific for the PDGF receptors (Fig. 5, middle and lower panel). However, after 2 h of stimulation the levels of both PDGF receptors were significantly down-regulated.

Effect of PDGF AA on T265-2c cell proliferation

The neurofibrosarcoma-derived Schwann cells, contrary to normal Schwann cells, displayed high levels of PDGF receptor α and β expression. It is known that PDGF BB can bind with high affinity to PDGF receptor α and to PDGF receptor β . Therefore PDGF BB could induce mitogenic signals through either receptor. PDGF AA, however, is known to bind and activate PDGF receptor α specifically. It was therefore of interest to evaluate the mitogenicity of PDGF AA for the neurofibrosarcoma-derived cell lines. T265-2c cells were cultured in serum-free media in the presence of 50 ng/ml PDGF AA or PDGF BB for 3 to 9 days. PDGF BB induced very robust growth of the T265-2c cells (Fig. 6A). Comparatively, PDGF AA had only a weak mitogenic effect on T265-2c cells. The growth rate of the PDGF AA-stimulated cells was approximately three times lower than the growth rate of the PDGF BB-stimulated cells. PDGF BB-induced proliferation was dose dependent (Fig. 6B), with a maximum effect observed at 50 ng/ml, as shown before. PDGF AA dose-response analysis revealed a plateau between 0.1 ng/ml and 10 ng/ml PDGF AA and a second slightly higher plateau above 50 ng/ml (Fig. 6B).

To understand why PDGF AA mitogenicity for T265-2c cells was only moderate, we analyzed PDGF AA-induced activation of PDGF receptors (Fig. 6C). Levels of tyrosyl-phosphorylated PDGF receptors induced by PDGF AA were very low when compared to PDGF BB-induced tyrosyl-phosphorylation. Upon stimulation with PDGF AA, the level of activated PDGF receptors was increased within 1 min and back to baseline within 30 min.

The previous results indicated that PDGF BB mitogenic activity is transduced by PDGF receptor β . To find out unambiguously which PDGF receptor is activated by PDGF AA or PDGF BB, immunoprecipitation experiments were performed using specific antibodies for PDGF receptor α or β (Fig. 7). T265-2c cells, incubated with 50 ng/ml of PDGF AA or PDGF BB for 10 min, were immunoprecipitated with an antibody to PDGF receptor α or to PDGF receptor β and the level of tyrosyl-phosphorylated PDGF receptor analyzed by immunoblotting. PDGF BB-induced cells expressed high levels of tyrosyl-phosphorylated PDGF receptor β , but only low levels of activated PDGF receptor α (Fig. 7, upper panel). PDGF AA induced the formation of low levels of activated PDGF receptor α , but no tyrosyl-phosphorylated PDGF receptor β , confirming the specificity of PDGF AA for the α receptor and providing an explanation for the modest mitogenicity of PDGF AA. Differences in the levels of tyrosyl-phosphorylated proteins were actually due to changes in the levels of phosphorylation and not to differences in the amount of protein immunoprecipitated (Fig. 7, lower panel).

DISCUSSION

Despite the identification of the *Nf1* gene as a primary target in NF1, the mechanisms leading to the formation of neurofibromas and neurofibrosarcomas remain unclear. In this study, we show that two neurofibrosarcoma-derived Schwann cell lines display radically modified growth factor receptor expression compared to Schwann cell lines not derived from NF1 patient or to adult Schwann cells (Hardy et al., 1992; Eccleston et al., 1993), resulting in functional changes in their mitogenic response to growth factors. PDGF BB induces a strong proliferative response from the two neurofibrosarcoma-derived Schwann cell lines. Mitogenicity of PDGF BB is transduced essentially through PDGF receptor β , which is highly increased in these two cell lines, compared to non-NF1 Schwann cell lines. PDGF receptor α expression is also increased in the neurofibrosarcoma-derived Schwann cells, but does not seem to play an important role in neurofibrosar-

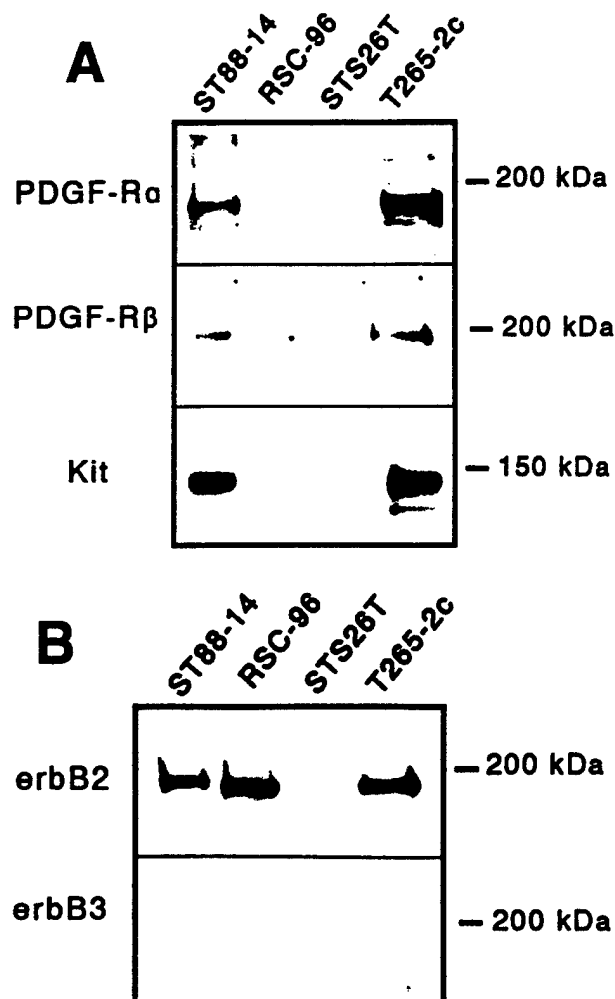


Fig. 4. Expression of PDGF receptors, Kit and erbB2/3 receptors in neurofibrosarcoma-derived Schwann cell lines. Cell lysates (50 μ g/lane) from neurofibrosarcoma-derived Schwann cell lines (ST88-14 and T265-2c), the schwannoma-derived cell line STS-26T, and the rat Schwann cell line RSC-96 were analyzed by immunoblotting by using antibodies specific for PDGF receptor α (A, upper panel), PDGF receptor β (A, middle panel), Kit (A, lower panel), erbB2 (B, upper panel), or erbB3 (B, lower panel).

coma-derived Schwann cell proliferation. Indeed, the low levels of activated PDGF receptor α observed upon PDGF AA (or PDGF BB) stimulation are insufficient to induce an effective proliferative response from the neurofibrosarcoma-derived Schwann cells. FGF2 also appears to be mitogenic for the neurofibrosarcoma-derived Schwann cells, whereas NDF β has no effect on neurofibrosarcoma-derived Schwann cell proliferation, due to a dramatic decrease in erbB3 expression.

PDGF BB and FGF2 are known to be potent mitogens for rat Schwann cells, especially in the presence of elevated intracellular cAMP (Davis and Stroobant, 1990), but are not known to induce proliferation of human Schwann cells (Li et al., 1996). Indeed, neuregulins are the only potent mitogens for human Schwann cells (Levi et al., 1995). Neurofibrosarcoma-derived Schwann cells proliferate in response to PDGF BB and FGF2 but are

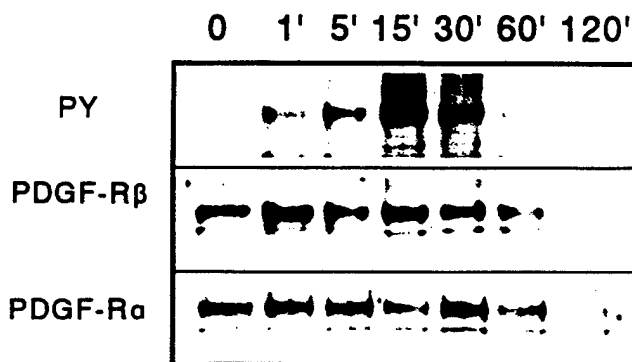


Fig. 5. Time-course of PDGF BB-induced activation of PDGF receptors. T265-2c cells were stimulated with 50 ng/ml PDGF BB. At the indicated time points, cells were lysed and proteins analyzed by immunoblotting using antibodies specific for phosphotyrosine (upper panel). Levels of PDGF receptors were estimated by probing the membrane with antibodies to PDGF receptor β (middle panel) and PDGF receptor α (lower panel).

not affected by the addition of NDF β , a neuregulin isoform which is a potent mitogen for cultured rat and human Schwann cells (Raabe et al., 1997; Lopez and De Vries, unpublished observations). This radical change in responsiveness to the different growth factors can be readily explained, at least in the case of PDGF BB and NDF β , by a change in the repertoire of growth factor receptors expressed by the neurofibrosarcoma-derived Schwann cells. The NF1-derived cell lines overexpress PDGF receptors compared to a Schwannoma-derived cell line or a rat Schwann cell line, cultured in the same conditions. Studies of PDGF receptor expression during sciatic nerve development show that, while PDGF receptor β is expressed at relatively high levels in neonatal Schwann cells, it is expressed at low levels in the adult. There is little or no expression of PDGF receptor α in adult Schwann cells (Hardy et al., 1992; Eccleston et al., 1993). Therefore inappropriate expression of PDGF receptors, especially PDGF receptor β , recapitulates an early step of development corresponding to active proliferation of Schwann cells.

NDF β does not stimulate the growth of the neurofibrosarcoma-derived Schwann cell lines due to the loss of the high affinity neuregulin receptor, erbB3. Only the schwannoma-derived cell line shows a strong decrease in erbB2 receptor expression. Actually, erbB2 has no known ligand but acts as preferred heterodimerization partner for erbB3 (Tzahar et al., 1997). ErbB3 has high affinity for neuregulins, but by itself is devoid of kinase activity (Guy et al., 1994). Upon binding to neuregulin, erbB2/erbB3 form a complex with high ligand affinity and potent signaling activity. Thus, the loss of one of the two partners in the erbB2/erbB3 complex (in this case the loss of erbB3) is sufficient to prevent NDF β -induced activity. Changes in neuregulin receptor expression do not seem to be related to NF1 specifically, since the schwannoma-derived cell line and the rat transformed cells also show strongly reduced erbB3 expression. Decreased erbB3 expression may therefore represent a general step in the process of Schwann cell malignant transformation. It remains unclear why a molecule which is central for Schwann cell

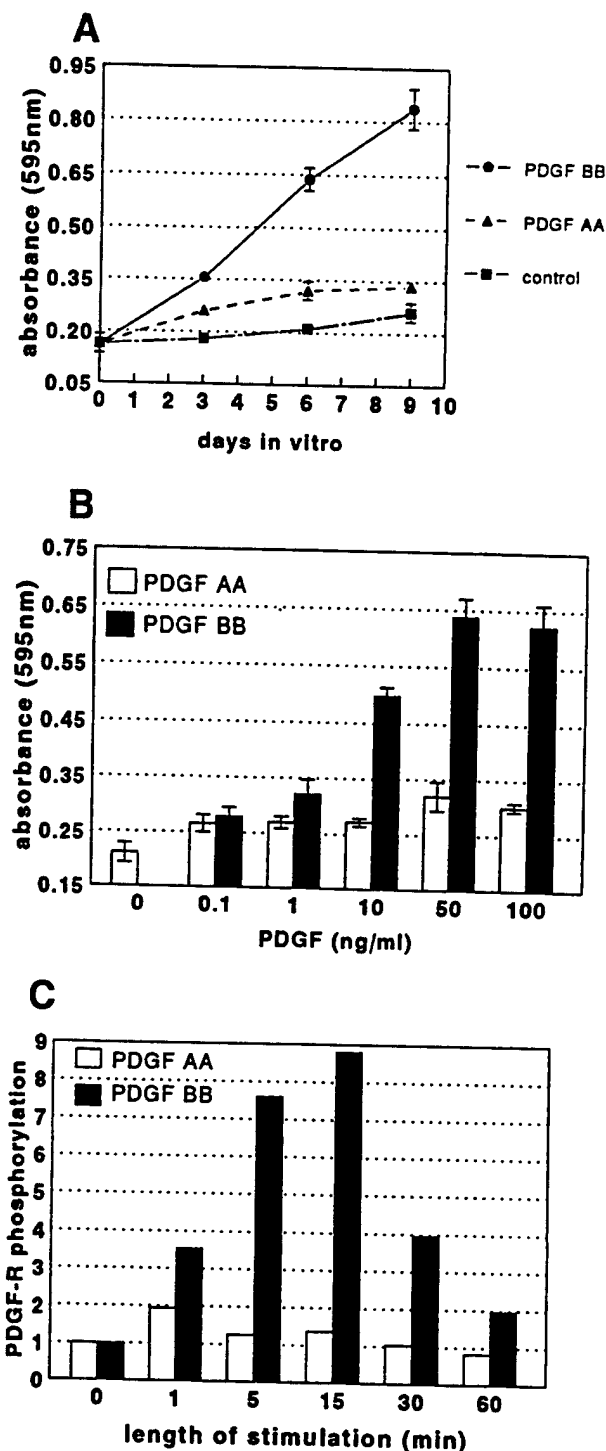


Fig. 6. PDGF AA is a weak mitogen for the T265-2c cell line. **A:** T265-2c cells were cultured in the presence of 50 ng/ml PDGF AA or PDGF BB and cell number evaluated over time as described in Figure 2. **B:** T265-2c cells were cultured for 6 days in the presence of increasing amounts of PDGF AA or PDGF BB and cell number evaluated as described in Figure 2. **C:** Activation of PDGF receptors by PDGF AA and PDGF BB was evaluated in parallel as described in Figure 5. Levels of tyrosyl-phosphorylated PDGF receptor was estimated by densitometric analysis of the band immunostained with the antibody to phosphotyrosine and expressed in arbitrary units. The experiment was repeated twice with similar results.

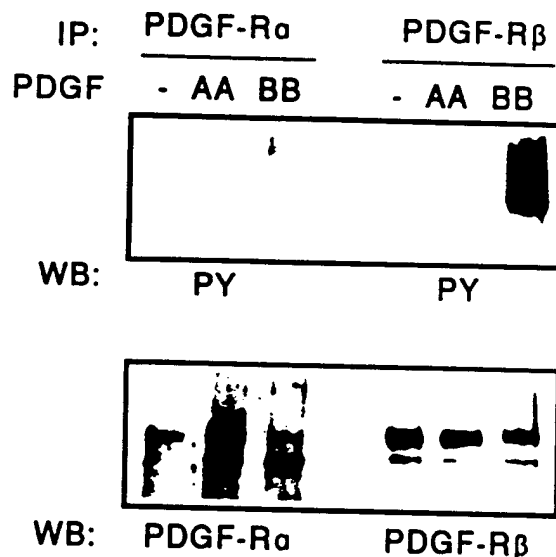


Fig. 7. High levels of activated PDGF receptor β in the T265-2c cell line upon PDGF BB stimulation. T265-2c cells were cultured in control serum-free medium (C) or in the presence of 50 ng/ml of PDGF AA or PDGF BB. After 10 min, cells were lysed and PDGF receptor α or β were immunoprecipitated (IP) using specific antibodies. Immunoprecipitated proteins were analyzed by immunoblotting (WB) using an anti-phosphotyrosine antibody (PY, upper panel). The membrane was stripped and the appropriate part of the membrane reprobed with an antibody to PDGF receptor α or β (lower panel).

survival and proliferation during development (Morissey et al., 1995; Grinspan et al., 1996) would be eliminated during the transformation process, which generally involves both increased cell growth and decreased cell death.

It is noteworthy that Schwann cells from the *Nf1* knock-out mouse also display decreased responsiveness to another member of the neurotrophin family, glial growth factor 2 (Kim et al., 1995). But *Nf1* knock-out mouse Schwann cells show decreased responsiveness to FGF2 as well (Kim et al., 1997), whereas neurofibrosarcoma-derived Schwann cell proliferation is increased by FGF2. Another difference between the two types of cells is the effect of forskolin, which by itself induced hyperplasia of the *Nf1* knock-out mouse Schwann cells, but had no effect on proliferation of the neurofibrosarcoma-derived Schwann cells. This difference in behavior between the two *Nf1* knockout cells and the neurofibrosarcoma-derived cells can have several explanations, such as the difference in species, the culture conditions and the status of the transformation process. Indeed, suppression of neurofibromin expression by targeted mutations in the knockout model reproduces early events in the pathologic process leading to the development of NF1. Neurofibrosarcoma-derived cells most probably acquired multiple mutations during their progression toward the malignant phenotype, which may be responsible for the aberrant expression of growth factor receptors or insensitivity to forskolin. Interestingly, these two events may be linked, since it has been shown that forskolin potentiates the mitogenic effect of PDGF BB for Schwann cells, through the induction of PDGF receptor expression (Weinmaster and Lemke, 1990). The fact that, contrary to what

was observed in human and rat Schwann cell cultures, forskolin does not potentiate the mitogenicity of neuroregulins, PDGFBB, or FGF2 for neurofibrosarcoma-derived Schwann cells may indicate that cAMP levels in these cells are chronically elevated, which may in turn result in higher expression of growth factor receptors.

PDGF BB is the most potent mitogen for the neurofibrosarcoma-derived Schwann cells. PDGF BB is known to bind both PDGF receptor α and PDGF receptor β (Seifert et al., 1989; Heldin and Westermark, 1990). Immunoprecipitation experiments indicate that, in the neurofibrosarcoma-derived Schwann cells, PDGF BB stimulation results in high levels of activated PDGF receptor β , but low levels of activated PDGF receptor α . PDGF AA, which is known to bind specifically to PDGF receptor α (Seifert et al., 1989), generates similarly low levels of activated PDGF receptor α , and a weak mitogenic response from the neurofibrosarcoma-derived cells. Thus, the increase in PDGF receptor α expression observed in the neurofibrosarcoma-derived Schwann cells may not be large enough to trigger the intracellular signaling pathways involved in proliferation. An estimation of the numbers of PDGF receptors in the T265-2c cell line using NIH-3T3 cells (a commonly used cell type in PDGF receptor studies) as a reference indicate a ratio of PDGF receptor β to PDGF receptor α of 2 to 1. Thus, the lower ratio of PDGF receptor α to PDGF receptor β may explain, in part, the lower level of activation and proliferation induced by PDGF AA. But, the modest effect of PDGF AA on T265-2c cell proliferation may also reflect PDGF receptor α lesser ability to trigger some signaling pathways (Eriksson et al., 1992; Heidaran et al., 1993).

It is now accepted that neurofibrosarcoma initiation and growth are probably the results of multiple sequential mutations involved in the progression toward a malignant phenotype. In addition to mutations in *Nf1*, loss-of-function mutations in tumor suppressor genes such as p53 (Ducatman et al., 1986; Halling et al., 1996) or gain of function mutations in oncogenes such as PDGF receptors, FGF receptors, Kit (Badache et al., 1998), or c-MET (Rao et al., 1997), as well as epigenetic events are likely to participate to the formation of Schwann cell tumors in NF1. Identification of the factors influencing the progression of Schwann cells toward a hyperplastic phenotype could provide new therapeutic strategies for NF1.

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SHORT REPORT

Expression of Kit in neurofibromin-deficient human Schwann cells: role in Schwann cell hyperplasia associated with Type 1 Neurofibromatosis

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Type 1 Neurofibromatosis (NF1) is characterized by the formation of neurofibromas, benign tumors composed mainly of Schwann cells, which can turn malignant to form neurofibrosarcomas. Neurofibromin, the protein product of the *Nf1* gene, is believed to act as a tumor suppressor, accelerating the conversion of the oncogene Ras to its inactive form. The absence of neurofibromin could therefore lead to higher Ras activity in Schwann cells, resulting in uncontrolled growth through a cascade of events not yet elucidated. We describe the abnormal expression of high levels of the Kit tyrosine kinase receptor in both NF1-derived Schwann cell lines and tissue, as compared to primary Schwann cells or schwannoma-derived cells. High levels of Kit expression in the neurofibrosarcoma-derived Schwann cells correlate with a decrease in neurofibromin expression. Using inhibitors of tyrosine kinase receptors, we found that proliferation of the neurofibrosarcoma-derived cells is dependent upon activation of a subclass of tyrosine-kinase receptors. The proliferation of these cells is not dependent upon an autocrine loop involving typical Schwann cell mitogens. Finally, the proliferation of the neurofibrosarcoma-derived Schwann cells can be increased by stimulation with Kit ligand. These data implicate Kit as one of the components leading to the Schwann cell hyperplasia observed in NF1.

Keywords: neurofibromatosis; Schwann cells; *c-kit* proto-oncogene; stem cell factor; growth factors

Neurofibromatosis Type 1 (NF1) is a human genetic disorder affecting approximately one in 3000 individuals that manifests with various phenotypic features, including neurofibromas, café-au-lait spots, axillary freckling, Lisch nodules, optical gliomas, skeletal abnormalities and learning disabilities (Riccardi, 1991). Neurofibromas are benign peripheral nerve sheath tumors, which are comprised primarily of Schwann cells, but also contain fibroblasts, perineurial cells and mast cells embedded in an abundant extracellular matrix. Patients with NF1 are at risk for development of malignant Schwann cell tumors, called neurofibrosarcomas, which often arise from a preexisting neurofibroma (Ducatman *et al.*, 1986; Halling *et al.*, 1996). The *Nf1* gene, mutated in NF1, was

identified by positional cloning (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990) and its protein product, neurofibromin, was found to be expressed at highest abundance in neurons, Schwann cells and oligodendrocytes (Datson *et al.*, 1992). Because the *Nf1* gene shares sequence homology with genes coding for Ras GTPase activating proteins (Buchberg *et al.*, 1990; Xu *et al.*, 1990), it was hypothesized that in the absence of functional neurofibromin, Ras deregulation could lead to tumor formation in NF1 (Basu *et al.*, 1992; DeClue *et al.*, 1992). However, introduction of activated Ras into normal Schwann cells resulted in growth arrest (Ridley *et al.*, 1988) and Schwann cells from neurofibromin deficient mice exhibit activation of Ras and inhibition of cell proliferation (Kim *et al.*, 1995). Therefore mutation in NF1 itself cannot explain NF1-Schwann cell hyperplasia and other factors underlying the development of NFS still need to be investigated.

Stem cell factor (SCF) is a growth factor required for the development of hematopoietic stem cells, primordial germ cells and melanocytes (Williams *et al.*, 1992). We have previously shown that normal Schwann cells, as well as a neurofibrosarcoma (NFS)-derived cell line express SCF (Ryan *et al.*, 1994). The receptor for SCF is the protein product of the proto-oncogene *kit*, which belongs to the platelet-derived growth factor (PDGF) subgroup of tyrosine kinase receptors (Yarden *et al.*, 1987). Interestingly, Kit was not found in normal Schwann cells, but was expressed in the NFS-derived Schwann cell line, raising the possibility of an autocrine stimulated growth (Ryan *et al.*, 1994). Therefore, it was of interest to further investigate the role of Kit in the development of Schwann cell tumors associated with NF1. In this study, we analysed both Kit and neurofibromin expression in primary Schwann cells, in Schwann cell lines derived from NF1-associated tumors and in a Schwann cell line derived from a sporadic schwannoma. We also investigated the possible contribution of tyrosine kinase receptor activation, in general, and Kit activation, in particular, to the hyperproliferative process leading to Schwann cell hyperplasia in NF1.

In order to show that expression of high levels of Kit is a feature specific for Schwann cells derived from NF1 patients, we evaluated the expression of Kit in four different Schwann cell lines, by Western blotting. Three of the cell lines, ST88-14, T265-2c and NF1T were derived from neurofibrosarcomas resected from patients diagnosed with NF1. The fourth cell line, called STS-26T, was isolated from an isolated grade III malignant schwannoma in an individual without NF1. Immunocytochemical analysis revealed that the overall

pattern of expression for Schwann cell markers is consistent with a Schwann cell origin for each of the four tumor-derived cell lines (Ryan *et al.*, 1994; Klein and De Vries, submitted for publication). Western blotting analysis confirmed that the tumor-derived cell lines expressed significant levels of CNPase (2', 3'-cyclic nucleotide 3'-phosphohydrolase) and laminin (data not shown). The ST88-14 and T265-2c cell lines displayed high levels of Kit protein compared to the STS-26T and NF1T cells (Figure 1a). Primary rat Schwann cells showed little Kit expression. The NF1 phenotype has been linked to mutations in the *Nf1* gene. Therefore we analysed the expression of neurofibromin in the four cell lines (Figure 1b). Human neurofibromin appeared as a 250 kDa doublet band on Western blots. The schwannoma derived cell line, STS-26T, showed levels of neurofibromin comparable to normal Schwann cells. The two NFS-derived Schwann cell lines expressed very low levels (ST88-14) or no detectable levels of neurofibromin (T265-2c). Surprisingly, NF1T cells, although derived from a patient diagnosed with NF1, displayed levels of neurofibromin comparable to cultured rat Schwann cells or to the STS-26T cell line. Thus, Schwann cell lines expressing neurofibromin did not express appreciable levels of Kit, whereas cell

lines deficient in neurofibromin showed high expression of Kit protein.

To evaluate the state of Kit activation in the cell lines expressing high levels of Kit, proteins were immunoprecipitated with an anti-Kit antibody, then analysed using an anti-phosphotyrosine antibody. Two proteins, a 145 kDa protein corresponding to Kit and a smaller protein of approximately 80 kDa, were immunoprecipitated from both NFS-derived Schwann cell lines (Figure 1c, lanes 1 and 2). The astrogloma cell line A172 (lane 3), which has high levels of Kit expression, was used as a positive control. When used in Western blotting analysis of whole cell lysates, the same anti-Kit antibody recognized predominantly the 80 kDa protein. Both Kit and the smaller protein immunologically related to Kit were tyrosyl-phosphorylated (Figure 1d). A 100 kDa phosphotyrosylated protein was also observed, but was immunoprecipitated by non-specific rabbit immunoglobulins as well (lane 4).

Kit expression in the NFS-derived Schwann cells was also analysed by immunocytochemistry (Figure 2a). ST88-14 cells, which can appear as bipolar spindle-shaped cells or pleiomorphic flattened cells, showed high levels of Kit expression throughout the cell bodies and processes. While the level of cytosolic Kit expression was relatively homogeneous within the cell population, some cells also displayed a strong nuclear staining. High expression of Kit was not only observed *in vitro* in the NFS-derived cell lines, but also *in vivo*, as revealed by immunohistochemistry of fresh tissue sections from an NF1 tumor. The tumor, identified as a plexiform neurofibroma, was first analysed using an antibody against the Schwann cell marker, S100. The anti-S100 antibody stained elongated, spindle shaped cells, with a morphology characteristic of the Schwann cells present in neurofibromas (not shown). The Kit antibody had a wider spectrum of staining. In addition to staining the cell bodies and processes of the S100 reactive Schwann cell-like cells, Kit antibody also stained large round cells, which have a morphology consistent with mast cells (Figure 2b).

Kit is present and tyrosyl-phosphorylated in the NF1-derived cell lines. If Kit is involved in the proliferation of the NFS-derived Schwann cells, then inhibition of the tyrosine kinase activity in the cells

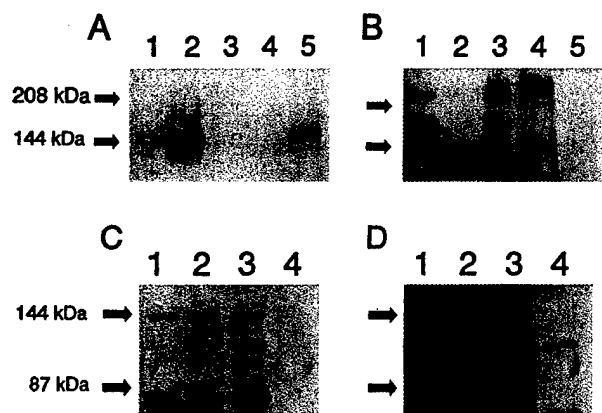


Figure 1 Upper panel: Expression of Kit in NFS-derived cell lines. Primary rat Schwann cells (lane 1), NFS-derived cell lines ST88-14 (lane 2), NF1T (lane 3) and T265-2c (lane 5) and the schwannoma cell line STS-26T (lane 4) were examined for Kit expression (a) and neurofibromin expression (b). NFS-derived cell lines deficient in neurofibromin (MW 250 kDa) expressed high levels of Kit (MW 145 kDa). Lower panel: Tyrosyl-phosphorylation of Kit in NFS-derived cell lines. Cell lysates from the ST88-14 (lane 1) and T265-2c (lane 2) cell lines and from the glioma cell line A172 (lane 3) were immunoprecipitated with an anti-Kit antibody; lane 4: ST88-14 cell line immunoprecipitated with non-specific rabbit immunoglobulins. Immunoprecipitated proteins were immunoprobed with an anti-Kit antibody (c) or an anti-phosphotyrosine antibody (d). For immunoprecipitation, cells cultured in serum-free media were lysed and incubated overnight in the presence of a preformed complex of Protein A-agarose/anti-Kit antibody (C-19, Santa-Cruz Biotechnology) at 4°C. The cell lysates and the immunoprecipitated proteins were analysed by electrophoresis in a 7.5% polyacrylamide gel and transfer to a PVDF membrane (Dupont, NEN). After blocking with a 5% nonfat dry milk solution, the PVDF membrane was probed with the appropriate primary antibody (anti-Kit Ab-1 from Oncogene Science, anti-neurofibromin from Santa Cruz Biotechnology or anti-phosphotyrosine from Transduction Laboratories). The antibody was detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Dupont-NEN). Primary rat Schwann cells were prepared according to Brockes and colleagues (1979)

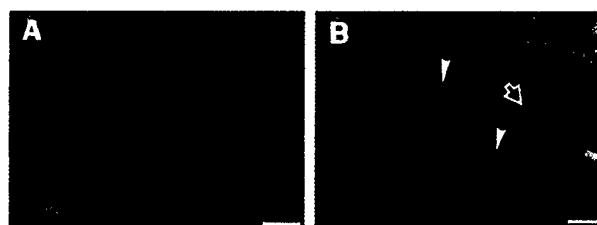


Figure 2 Immunocytochemical detection of Kit in the NFS-derived Schwann cell line ST88-14 (a) and in fresh frozen sections (15 μ m) of a plexiform neurofibroma (b). Cells and tissue were incubated with an anti-Kit antibody (Santa-Cruz Biotechnology) and visualized using a rhodamine-conjugated secondary antibody. Immunocytochemistry was performed as described before (Lee *et al.*, 1996). In the tumor, Kit is present in both large round cells, probably mast cells (arrow) and spindle-shaped, Schwann-like cells with extended processes (arrowheads). In the absence of primary antibody, cells and tissue sections showed no fluorescence. Bar is 10 μ m

should decrease their proliferation. The NFS-derived cell lines, ST88-14 and T265-2c, and the A172 line were cultured in the presence of increasing concentrations of tyrosine kinase inhibitors, in serum-free media. Tyrosine kinase inhibitors acting either through competitive inhibition of ATP binding (Genistein) or by competitive inhibition of substrate binding to the kinase domain (Tyrphostin A9 and Tyrphostin A25)

were tested. Tyrphostin A9, a tyrosine kinase inhibitor specific for PDGF-like tyrosine kinase receptors, decreased the rate of NFS-derived Schwann cell proliferation in a dose-dependent manner with an ED50 of approximately 1.5 μM (Figure 3a), close to its Ki/IC_{50} (Levitzki and Gilon, 1991). Tyrphostin A25 and Genistein, with respective ED50 of 80 μM and 25 μM , were efficient inhibitors of cell proliferation only at concentrations largely higher than their published Ki/IC_{50} (Gazit *et al.*, 1989), probably targeting a wide range of tyrosine kinase receptors. To demonstrate that tyrosine phosphorylation was actually inhibited by the tyrosine kinase inhibitors, ST88-14 cells were collected after 3 days of treatment with the tyrosine kinase inhibitors. Proteins were then analysed by Western blotting using an anti-phosphotyrosine antibody and the lanes were scanned to evaluate the relative amounts of tyrosyl-phosphorylated proteins. We observed a general decrease in the level of tyrosine phosphorylated proteins in cells cultured in the presence of Genistein (45% decrease), Tyrphostin A9 (85% decrease) and Tyrphostin A25 (90% decrease) which could be correlated to the decrease in cell proliferation observed in the presence of these inhibitors (Figure 3d).

Proliferation of many tumor cell lines is driven by an autocrine loop, involving activation of a growth factor receptor by its ligands expressed by the same cells. To determine if a Kit/SCF autocrine loop was responsible for the NF1 cell line proliferation, cell growth was evaluated in the presence of neutralizing antibodies to SCF (R&D systems) or to Kit (clones 1.D9.3D6, Boehringer Mannheim Corporation or K44.2, Sigma Bioscience). These antibodies have been used to interfere with SCF activity in various systems and in particular to disrupt the autocrine growth of breast carcinoma cell lines (Bleechman *et al.*, 1993; Hines *et al.*, 1995). Anti-SCF antibodies (used at concentrations as high as 50 $\mu\text{g}/\text{ml}$) had no significant effect on the proliferation rate of the NFS-derived Schwann cell lines grown in serum-containing or serum-free media. (Figure 4a). Similarly, both anti-Kit antibodies (used at concentrations as high as 25 $\mu\text{g}/\text{ml}$) failed to prevent the growth of the NFS-derived cell lines (data not

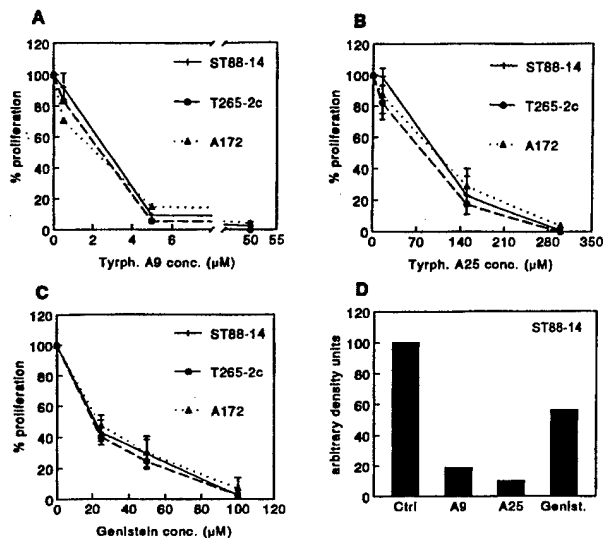


Figure 3 Effect of tyrosine kinase inhibitors on the proliferation of NFS-derived cell lines and the A172 glioma cell line. Cells (5000/well) were incubated in serum-free media for 3 days, in the presence of increasing amounts of Tyrphostin A9 (a), Tyrphostin A25 (b) or Genistein (c), all obtained from LC Laboratories. Cell number at 0 and 3 days was evaluated by the colorimetric MTT assay (Boehringer Mannheim Corporation). Absorbance was measured at 595 nm and growth expressed as the change in absorbance relative to untreated controls. Results were expressed as the mean \pm s.e. of triplicate wells ($n=3-5$). Microscopic examination showed little if any cytotoxicity of the compounds at the doses used in these experiments. (d) ST88-14 cells treated with 5 μM Tyrphostin A9, 150 μM Tyrphostin A25 or 50 μM Genistein as described above were lysed and the proteins analysed by Western blotting using an anti-phosphotyrosine antibody. Densitometric analysis of proteins was performed using the BioRad GS-670 densitometer and expressed relative to control (cells treated with vehicle)

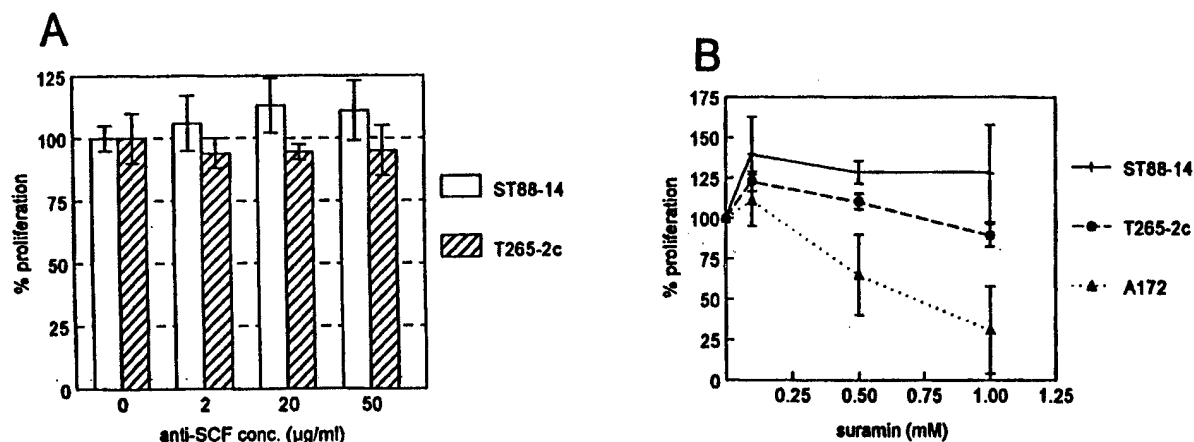


Figure 4 Effect of anti-SCF antibodies (a) and suramin (b) on the proliferation of NFS-derived cell lines and the A172 glioma cell line. Cells were incubated in the presence of increasing amounts of neutralizing antibodies to SCF (R&D Systems) or suramin (Calbiochem) in serum-free media for 3 days and proliferation expressed as in Figure 3. Suramin inhibits the proliferation of the A172 cell line, but has no significant effect on the proliferation of NF1-derived Schwann cell lines

shown). In order to evaluate the possibility of an autocrine loop involving other growth factors and their receptors, cells were cultured in the presence of suramin, a blocker of growth factor/growth factor receptor interactions with a broad range of action. Suramin was very effective in blocking the proliferation of the A172 cell line (Figure 4b), which is known to be largely dependent upon an autocrine loop involving PDGF/PDGF receptors (Fleming *et al.*, 1992; Sato and Nitta, 1994). Suramin did not decrease the growth of the NFS-derived Schwann cell lines. A limited increase in cell number compared to untreated cells was observed at low suramin concentrations (Figure 4b).

In order to directly test if Kit can assume a role in Schwann cell hyperplasia, the effect of SCF on proliferation of the four Schwann cell lines was evaluated (Figure 5a). Cells were cultured in the presence of 20 ng/ml SCF. After 4 days in culture the number of cells was evaluated relative to untreated cells. SCF produced a significant increase in the number of ST88-14 cells. The response of the T265-2c cell line, which expressed intermediate levels of Kit, to SCF was less robust but significant. In contrast, SCF was a poor mitogen for the NF1T and STS-26T cell lines. ST88-14 cells and NF1T cells were cultured over longer periods of time in the presence of increasing concentrations of SCF. SCF stimulated the proliferation of the ST88-14 cells in a dose-dependent manner, leading to a twofold increase in cell number over 10 days, relative to untreated cells (Figure 5b); the effect was maximal at 10 ng/ml and less at 100 ng/ml. SCF did not significantly stimulate the proliferation of the NF1T cell line (Figure 5c).

In this report, we have shown that the proto-oncogene Kit is highly expressed in NFS-derived cells deficient in neurofibromin, but expressed at low levels in primary Schwann cells or malignant Schwann cells with normal levels of neurofibromin expression. Kit expressed by the NFS-Schwann cells is functional and SCF stimulates the NFS-derived Schwann cells to proliferate. These results indicate that high expression of Kit in NFS-derived Schwann cells may be associated with the NF1-phenotype (i.e. neurofibromin deficiency) and that SCF stimulation of Schwann cell proliferation can contribute to the hyperplastic phenotype that leads to Schwann cell tumor formation in NF1 patients.

Kit is expressed at low levels in adult human Schwann cells (Ryan *et al.*, 1994, TJ Lopez and GH DeVries, unpublished data), but is expressed at high levels in the ST88-14 and T265-2c cell lines, two NFS-derived Schwann cell lines. Kit expression appears to be specifically associated with the NF1 phenotype and not, more generally, with a transformed phenotype. Indeed, the STS-26T cell line, a schwannoma-derived cell line from a patient without NF1, and an immortalized rat Schwann cell line (Ryan *et al.*, 1994) display very limited expression of Kit. The observation that the NF1T cell line, which is a NFS-derived cell line, does not display high levels of Kit could appear in contradiction with this hypothesis. But examination of neurofibromin expression in the different cell lines shows that the NF1T cell line, contrary to the other NFS-derived lines, expresses levels of neurofibromin comparable to normal cells. It has been shown that Schwann cell differentiation is

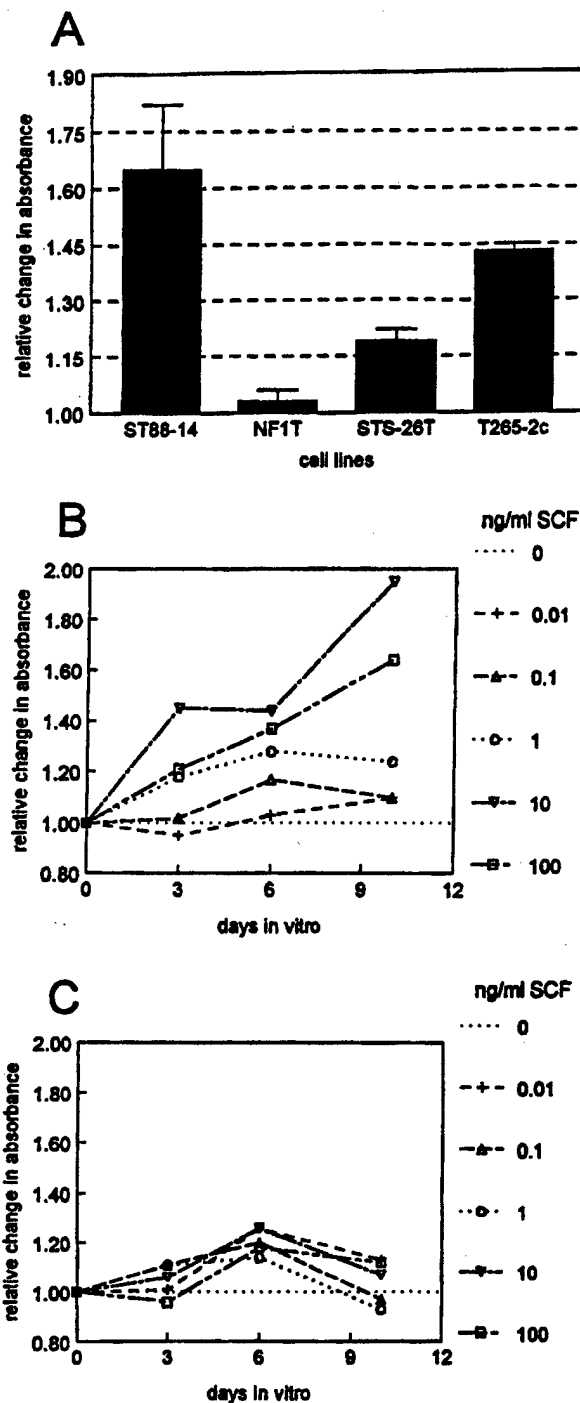


Figure 5 Effect of SCF on the proliferation of the NFS-derived cell lines and the schwannoma-derived cell line. (a) Cell lines were grown in the presence of 20 ng/ml recombinant human SCF (R&D Systems) in serum-free media for 4 days. Cell number was evaluated using an MTT assay and the proliferation expressed relative to untreated cells. ST88-14 cells (b) and NF1T cells (c) were grown in the presence of increasing amounts of SCF in serum-free media, for either 3, 6 or 10 days. Proliferation was expressed relative to untreated cells. Results are expressed as mean of six wells, s.e. did not exceed 10%

associated with increased expression of neurofibromin (Gutmann *et al.*, 1993). Recent studies from our laboratory show that among the four Schwann cell lines described in this study, the NF1T cell line bears the most resemblance to normal Schwann cells. The

NF1T cell line maintains a bipolar morphology characteristic of Schwann cells, has the highest level of CNPase activity (CNPase is a biochemical marker of myelin) and retains its ability to preferentially associate with axons (Klein and DeVries, submitted for publication). Because of its more differentiated phenotype, it is not surprising to observe the expression of neurofibromin in the NF1T cell line. However, we cannot exclude the possibility that neurofibromin expressed in the NF1T cells may present subtle mutations that cannot be resolved using polyacrylamide gel electrophoresis. A recent study described that neurofibromas, with normal levels of neurofibromin, displayed intermediate levels of Ras-GTP, compared to neurofibrosarcomas (Guha *et al.*, 1996). This intermediate phenotype may be insufficient to induce an increase in Kit expression. Thus, although only a limited number of NF1-cell lines have been tested so far, our data indicate that Kit is over-expressed in NFS-derived Schwann cells showing a reduction in neurofibromin. However, a direct relationship between the decrease in neurofibromin and the up-regulation of Kit expression still needs to be demonstrated.

Kit expressed in the NFS-derived cell lines is constitutively tyrosyl-phosphorylated, possibly due to its interaction with endogenously produced SCF. We were able to block the proliferation of the NFS-derived Schwann cell lines, using low doses of an inhibitor selective for the PDGF-receptor subclass of tyrosine kinase receptor. The growth of the NFS-cell lines was not significantly affected by the addition of suramin to the media. Suramin, a polyanionic drug, is thought to interfere with the binding of the growth factors to low affinity, heparan-sulfate proteoglycan receptors. Suramin has been used in many instances to block autocrine growth loops due to PDGF, fibroblast growth factor and insulin-like growth factor, which are all well known mitogens for Schwann cells. It has been shown recently that suramin inhibits the responsiveness of Schwann cells to glial growth factor, the most potent Schwann cell mitogen (Sudhalter *et al.*, 1996). Because of the lack of effect of suramin on the proliferation of the NFS-derived cells, it is unlikely that any of these growth factors contribute to the autocrine growth of the NFS-derived cells. We were unable to interrupt the NFS-derived Schwann cell proliferation using antibodies specific for Kit and SCF. One possibility is that the SCF/Kit interaction takes place intracellularly. Even though the actual mechanism remains unclear, it has been shown that a PDGF/PDGF receptor autocrine growth loop can take place even when PDGF was modified so that it could no longer be secreted extracellularly (Keating and Williams, 1988). It is also believed that a large part of the interactions between SCF and Kit in breast carcinoma cell lines and tumors occurs intracellularly (Hines *et al.*, 1995). It is also possible that the 80 kDa protein represents a truncated, constitutively active form of Kit. Finally, it has recently been shown that, in human astrocytes, high levels of Kit expression in the absence of its ligand resulted in apoptotic cell death (He *et al.*, 1997).

Experiments evaluating the effect of SCF on the Schwann cell lines show unambiguously that Kit activation stimulates the proliferation of the NFS-derived cells. However, the mitogenicity of SCF for the NFS-derived lines, and for the T265-2c cell line in particular, is only moderate. This could be explained by the lower levels of Kit expression of this cell line (relative to the ST88-14) and the fact that a significant proportion of Kit may be interacting with endogenous SCF, limiting the response elicited by exogenous SCF. Similar observations were reported in breast carcinoma cells, co-expressing SCF and Kit; only high levels of Kit expression allow a growth response to an excess of SCF (Hines *et al.*, 1995). The dose required to elicit maximal cell growth in our study is relatively low, possibly due to the fact that Kit binding sites are already partially occupied by endogenous SCF. In the hemopoietic system, SCF alone has a very modest effect on the proliferation of early hemopoietic precursors but it markedly potentiates the effect of other growth factors, such as interleukin 3, erythropoietin, GM-CSF and G-CSF (McNiece *et al.*, 1991). Thus, it is possible that SCF may act in cooperation with other factors to stimulate NFS-derived Schwann cell proliferation. In that context, it will be interesting to test the effect of factors that increase intracellular cyclic AMP levels, as elevating cyclic AMP levels in *Nf1*-deficient mouse Schwann cells results in Schwann cell hyperplasia (Kim *et al.*, 1997).

We have shown that Kit is expressed at high levels in NFS-derived cells displaying decreased neurofibromin expression and that Kit activation by SCF can induce a proliferative response in NFS-derived cells. In the tumors, Kit may be activated by endogenous SCF produced by the Schwann cell-like cells themselves. However, *in vivo*, exogenous sources of SCF such as the fibroblasts present in the tumors and circulating SCF from the serum can contribute to Kit activation. The respective contributions of the various sources of SCF to Schwann cell hyperplasia in the neurofibrosarcomas still need to be investigated, as does the possible involvement of co-factors, produced by other cell types present in the tumors (fibroblasts, mast cells) which could potentiate SCF mitogenicity towards Schwann cells. In turn, a better understanding of the cellular mechanisms leading to the abnormal proliferation of Schwann cells in neurofibromas and neurofibrosarcomas will allow the design of new therapeutic strategies for NF1.

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**NF1 Schwann cells have elevated cAMP levels and increased
secretion of prostaglandin PGE₂**

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Abstract

Neurofibromatosis Type 1 (NF1) is a human genetic disorder affecting approximately 1 in 3000 individuals. Characteristics of this disease are benign neurofibromas and malignant neurofibrosarcomas primarily made up of Schwann cells. Although the lack of neurofibromin leads to elevated Ras activities contributing to Schwann cell hyperplasia, it has recently been appreciated that Schwann transformation requires additional intracellular abnormalities. We now report that NF1 Schwann cells have 2 fold higher cAMP levels than normal human adult Schwann cells. PCR analysis of normal adult human Schwann cells reveals adenylyl cyclase (AC) mRNA for types III, IV, and IX. Similar analysis of NF1 Schwann cells shows all the isoforms in normal adult human Schwann cells, but additionally the NF1 Schwann cells express AC mRNA of types II, V, VII, and VIII. Increased cAMP levels may be the results of prostaglandins secreted by Schwann cells themselves. In support of this view, we find that NF1 Schwann cells express higher levels of cPLA₂ and Cox-2 than control cells. PCR analysis reveals that NF1 Schwann cells express mRNA for EP2 and EP4 prostaglandin receptors while normal human Schwann cells only express the EP2 receptor. Interestingly, the addition of exogenous prostaglandins to NF1 Schwann cells induces further increases of cAMP levels and also induces the proliferation NF1 Schwann cells. The proliferation of NF1 Schwann cells in response to PDGF BB decreased by Cox-2 and PKA inhibitors. These results are consistent with the view that aberrant cAMP signaling and elevated prostaglandin metabolism in NF1 Schwann cells contribute to tumor formation in NF1 patients. (Supported by US Army Medical Research and Material Command; Contract grant number: DAMD17-98-8607).

Introduction

Neurofibromatosis type 1 (NF1) is an inherited disease, which is diagnosed by many symptoms including neurofibromas and café-au-lait spots (Friedman et al., 1999). A prominent feature of NF1 is the formation of benign Schwann cell tumors of peripheral nerve sheath called neurofibromas. In some instances, NF1 patients develop lethal malignant peripheral nerve sheath tumors, which are neurofibrosarcomas. The tumors of this genetic disease are caused by defects of the NF1 gene resulting in the absence of its protein product, neurofibromin. Although the function of neurofibromin is not known, its loss leads to the elevated levels of the activated form of Ras (Ras-GTP) (DeClue et al., 1992). It has been previously reported that Ras-GTP levels are elevated in neurofibrosarcoma tumors

from NF1 patients and Schwann cells derived from mice lacking NF1 (Feldkamp et al., 1998; Kim et al., 1997). However, the link between the development of Schwann cells tumors and the loss of neurofibromin has not been clearly established.

As is the case with several types of cancer, aberrant expression of growth factor receptors may play a role in cellular transformation. Schwann cell lines derived from NF1 tumors express growth factor receptors that are not present in normal human Schwann cells. Badache et al. (1998a) have previously reported that Schwann cells overexpress c-kit receptors. In addition, the aberrant expression of the EGF receptors has been documented on the surface of NF1 Schwann cells (DeClue et al., 2000). Interestingly, NF1 Schwann cells lacks Erb-B2 receptors, which are activated in response to neuregulin, the most potent growth factor for normal Schwann cells (Badache et al., 1999). Taken together, these results implicate aberrant growth factor receptor expression in the development of Schwann cell tumors in NF1 patients.

Since cAMP can regulate growth factor receptor expression in Schwann cells (Weinmeister et al., 1990) and cAMP is also a Schwann cell mitogen, intracellular levels of cAMP may play an important role in the proliferation of NF1 Schwann cells. Increased levels of intracellular cAMP brought about by forskolin stimulation of adenylyl cyclase causes enhanced Schwann cell proliferation when used in combination with growth factors such as, neuregulin β 1 and PDGF (Rahmatullah et al., 1998; Davis et al., 1990). It has been reported that sustained high levels of cAMP increase growth factor receptor expression leading to aberrant Schwann cell proliferation (Weinmaster et al., 1990). In this study, we report that NF1 Schwann cells express additional adenylyl cyclase isoforms and have higher basal levels of cAMP than that of normal Schwann cells. In addition, NF1 Schwann cells secrete higher levels of PGE₂ and express more EP receptors than normal adult Schwann cells. The addition of exogenous PGE₂ results in further elevation of intracellular cAMP and increased cell proliferation. Finally, we present evidence that the proliferation of NF1 Schwann cells stimulated by PDGF BB is mediated by Cox-2 and PKA.

Materials and Methods

Cell lines and Reagents

Recombinant platelet derived growth factor BB (PDGF BB) was purchased from R&D (Minneapolis, MN). H89, NS-398, and PGE2 were purchased from Biomol (Plymouth Meeting, PA), indomethacin from Sigma (St Louis, MO). Forskolin was purchased from Calbiochem (La Jolla, CA). The cell lines ST88-14, T265, STS 26T used in these studies were previously described (Badache et al., 1998). The 90-8 and 88-3 cell lines were obtained from Dr. Jeff DeClue have been previously described (DeClue et al. 1992). Cells were cultured as monolayers in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and 100ug/ml streptomycin, and maintained at 37°C in a humid atmosphere of 10% CO₂/90%air. For proliferation assay and western blotting, cells were serum starved overnight and were cultured in serum free media with PDGF BB alone or in combination with inhibitors.

Human Schwann cells

Primary human Schwann cell cultures were established as described by Wood et al., (2000) (Miami Project, University of Miami, Miami, Fla). Fresh human peripheral nerves (cauda equina) were obtained from Dr. Wood from transplant patients. For ten days, the nerve fragments were placed in DMEM containing 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), 2uM forskolin, 10nM NDF β 1 (Amgen Inc., Thousand Oaks, CA), 50U/ml penicillin and 0.05 mg/ml streptomycin (Sigma, St Louis, MO). Then the fragments were dissociated in DMEM containing 10% heat inactivated fetal bovine serum, 2uM forskolin, 10nM NDF, 50U/ml penicillin and 0.05 mg/ml streptomycin, 0.05% collagenase (Worthington Biochemicals, Freehold, NJ) and 0.25 % dispase (Boehringer Mannheim, Indianapolis, IN) in a erlenmyer flask. The nerve fragments were incubated at 37C in a 5% CO₂ incubator in the enzyme solution for 18h. After centrifugation, the cells were resuspended in fresh media and were plated in 100mm culture dishes in DMEM containing 10% heat inactivated fetal bovine serum, 2uM forskolin, and 10nM NDF β 1. In Schwann cells cultured without forskolin, the nerve fragments were incubated with DMEM containing 10% serum for 3 days. Then the nerve fragments were worked up as previously described in media containing 10% serum with no forskolin or neuregulin β 1. The cells were harvested 24 hours after plating.

NF1 tumors

NF1 tumors were obtained from patients diagnosed with NF1 and were removed by Dr. John Shea at Loyola University Medical Center.

Schwann cell culture

Primary Schwann cell cultures were established essentially as described by Brockes and colleagues (1979). Sciatic nerves were removed from 3-day rat pups, digested for 2 hours, in 0.03% collagenase (Serva) at 37°C, and triturated thoroughly to achieve dissociation. Cells were cultured as monolayers in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and 100ug/ml streptomycin, and maintained at 37°C in a humid atmosphere of 10% CO₂/90%air. Contaminating fibroblasts were inhibited by 72 hours treatment with 10uM cytosine arabinoside.

MTT assay

This assay involved a spectrophotometric analysis of the degradation of the bromide salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) by mitochondrial dehydrogenase enzymes yielding purple formazan crystals as a by-product of this reaction. After the incubation treatment, MTT labeling reagent (0.5mg/ml, Sigma, St Louis, MO) were added to Schwann cell cultures for 4 hours (humidified atmosphere, 5% CO₂, 37°C). Following overnight incubation with a solubilization solution (10% SDS in 0.01M HCL), the amount of solubilized formazan crystals were quantified at 595 nm using an automated microplate reader EL311sx (Bio-Tek Instruments, Inc., Winooski, VT). The increase in absorbance correlated with the number of cells.

Western blotting

Cells cultured in DMEM supplemented with 10% FCS or rat sciatic nerves were lysed in RIPA buffer (1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in phosphate buffer saline) containing a cocktail of protease inhibitors. Protein lysates were separated by electrophoresis in a polyacrylamide gel (Invitrogen) and transferred onto a PVDF membrane (Dupont, NEN, Boston, MA). After blocking with a 5% nonfat dry milk solution (5% milk in PBS containing 1% Tween, PBS-T) for 30 minutes, the PVDF membrane was incubated overnight in the presence of the primary antibody (anti-cPLA₂ from Santa Cruz Biotechnology or anti-Cox-2 from Transduction laboratory) at 4°C. After several washes in PBS-T, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Transduction Laboratories, San Diego, CA). Following additional washes with PBS-T, the immunoreactivity was detected by enhanced chemiluminescence (Dupont-NEN, Boston, MA).

REVERSE TRANSCRIPTION PCR

Total cellular RNA isolation and quantitation

Total RNA was isolated from cultured Schwann cells by a modification of the acid-method of Chomczynski and Sacchi (1987). Briefly, cells were washed with PBS and solubilized using Trizol reagent (Gibco-BRL). For each sample, 0.2 ml of chloroform were added per 1 ml of Trizol. After the samples were subjected to centrifugation at 12,000 x g for 15 min. at 4°C, the aqueous phase of each sample were transferred to a fresh tube. The RNA in the aqueous phase was precipitated by mixing 0.5 ml of isopropyl alcohol per 1 ml of Trizol reagent (used for the initial solubilization of cells). To enhance recovery of small amounts of RNA, 20 µg of glycogen were added as a carrier prior to isopropanol precipitation. Samples were subjected to centrifugation at 12,000 x g for 10 min. at 4°C and the RNA pellet obtained were redissolved in sterile water.

First strand cDNA synthesis by reverse transcriptase

Total cellular RNA were converted to cDNA in 20 µl reactions consisting of 1 µg RNA, 1xPCR buffer (Gibco BRL), 2.5 mM MgCl₂, 500mM dNTPs (Pharmacia), 10 mM DTT, 20 U RNAsin (Promega), 200 U Superscript II Rnase H⁻ reverse transcriptase (RT), and 2 µg random hexamer primers (Perkin-Elmer). Reactions were incubated at room temperature for 5 min. for primer annealing, then at 42°C for 60 minutes for reverse transcription. Reactions were terminated by a 5 min. incubation at 95°C, and RT products were used immediately.

Primer design for polymerase chain reaction (PCR)

Oligonucleotides of 21 base pairs were designed with the Oligo IV software using the gene sequence for each AC obtained from GenBank. The software was utilized for the analysis and design of the best upstream and downstream primers according to specific conditions, such as annealing temperatures, secondary structures, and several other factors. Oligonucleotides were synthesized for our laboratory by Gibco BRL.

Sizes and sequences for the upstream (UP) and downstream (DWN) each AC primers are as following:

ACI (1044bp):UP 5'-GGG GCT GCG TTT CTC TTG CTG-3'; DWN 5'-CAG GGC AAA AGC AAA CTC CAC-3'; ACII (581bp):UP 5'-GAG TTT GCT TTT GCC CTG GTA-3'; DWN 5'-GCG CTC CTT TTC CCT TTT GCT-3'; ACIII (774bp): UP 5'-CCT GGC GTC CCG TCT TTG ATG-3'; DWN 5'-CAG CCA GAA CCC CGC CTT TGT-3'; ACIV (559bp): UP 5'-TCG GGG ACT GTG AGA ACC TCT-3'; DWN 5'-TCA GCG TCC TCA TCA CCT ATG-3'; ACV (301bp):UP 5'-TGG GGC AAA ACT GTG AAC CTG-3'; DWN 5'-GCT GCT TCT GCC TTT GCC TAT-3'; ACVI (774bp): UP 5'-GCA CGG CTG TTG GAG TCT TCT-3'; DWN 5'-CAT TGT TGC CCT CCA GTT CCA-3'; ACVII (772bp):UP 5'-TCG CCT GCT CGG TCT TCC TGA-3'; DWN 5'-AGG CGG AAG GAG TTG AAG GAG-3';ACVIII (781):UP 5'-GCG GCT CGT GCT TTC TGT GCT-3'; DWN 5'-ATT ATC AAA GGG CAG TTC AGG-3'; ACIX(469): UP 5'-GGA GAA AAC GGA CGC CCA CTT-3'; DWN 5'-TGC GGG AAG CGA CAC CAG GAT-3'.

PCR conditions

After reverse transcription, PCR reactions were carried out in volumes of 50 μ l, using bulk mixes for multiple samples. PCR master mix consisted of 1xPCR buffer (Gibco BRL), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 3 U per final reaction Taq polymerase (Gibco BRL), and 0.3 μ M of each primer. Each sample consisted of 48 μ l of master mix and 2 μ l (50ng) of each completed RT reaction added to 0.7 ml microcentrifuge tubes. PCR reactions took place in a Hybaid thermocycler. The PCR profile was one cycle of 95°C for 5 min. for initial denaturation of cDNA/RNA hybrids, and one cycle of 80°C for 3 min. for the addition of Taq Polymerase; 32 cycles of 95°C for 45 sec., 62°C for 45 sec. and 72°C for 60 sec.; then a final extension cycle at 72°C for 5 min. to ensure complete synthesis of amplimers.

PCR product detection

Following PCR amplification, equivalent amount (10 μ l) of reaction products were separated by gel electrophoresis using 2% agarose gels in TAE buffer for 30 minutes. Amplimers were visualized following exposure of the gel to 2% ethidium bromide solution and were photographed under trans-illumination with a Polaroid MP4+ Camera system.

Cyclic AMP ELISA

Schwann cells were seeded onto 6 well culture plates at a density of 300,000 cells/well in DMEM with 10% FCS. Cells were serum deprived for 24 hours. Before treatment, Schwann cells were incubated with serum free media containing 250 μ M isobutyl methyl xanthine (IBMX) for 20 to 30 min to inhibit phosphodiesterase activity. After the media was aspirated, the cells were lysed with 0.1M HCl for 1 hour at room temperature on a rotary shaker. The lysate was collected in 1.5 ml centrifuge tubes and used immediately. Concentrations of cAMP were determined using the acetylated version of direct enzyme immunoassay kit (Assay Designs, Inc) according to the manufacturers instructions. The data are expressed as picomoles of cAMP per mg of protein. The concentrations of cAMP were determined in triplicate.

PGE₂ ELISA

Schwann cells were seeded onto 6 well culture plates at a density of 300,000 cells/well in DMEM with 10% FCS. Cells were serum deprived for 24 hours. After incubating the cells for 1 hour in fresh DMEM, the cells were lysed to determine protein concentrations for each condition and supernatants were collected into a 96 well-plate. Levels of PGE₂ were determined using the direct enzyme immunoassay kit (Assay Designs, Inc) according to the manufacturers instructions. Concentrations of PGE₂ were determined in triplicate wells and were normalized to protein levels. The levels of PGE₂ are expressed in pmole/ml/mg.

Results

Intracellular cAMP levels in NF1 and non-NF1 Schwann cell lines

Previous reports have shown that elevation of cAMP levels in normal Schwann cells lead to increase growth factor receptor expression, such as PDGF receptor (Weinmaster et al., 1990). Based on this observation, we wanted to investigate the basal levels of intracellular cAMP in NF1 derived Schwann cells, since elevated intracellular cAMP may lead to high PDGF receptor expression in NF1 cells. To determine basal levels of cAMP in Schwann cells, cells were incubated in serum free medium with a phosphodiesterase inhibitor (200uM IBMX). After a 20-minute incubation, cAMP concentrations measured by ELISA. The results show that three of the four NF1 cell lines expressed about 10pmoles of cAMP per mg of protein (**figure 26**). The normal rat Schwann cells expressed 2pmoles cAMP/mg and the adult human Schwann cells expressed 5 pmoles/mg. These results demonstrate that NF1 cells have higher basal cAMP levels than control cells.

Determination of adenylyl cyclases in Schwann cells

The higher cAMP levels of NF1 cells compared to the levels of normal Schwann cells may be related to the different subtypes of adenylyl cyclases each cell type expresses. We set out to determine the subtypes of adenylyl cyclases expressed by both NF1, non-NF1, and normal human adult Schwann cells. We performed RT/PCR using

specific primers for each 9 different subtypes of adenylyl cyclases. Normal human adult Schwann cells express mRNA for AC types III, IV, VII (weakly), and IX (**figure 27A**). A similar analysis conducted with NF1 Schwann cells revealed that they express the same AC isoforms as normal Schwann cells plus 3 additional AC subtypes: II, V, and VIII (**figure 27B**). Interestingly, the non-NF1 Schwann cells (STS26T) express similar AC subtypes as NF1 Schwann cells plus one additional AC subtype VI (data not shown). The AC mRNA expressed by Schwann cells are summarized in Table 1. The increased AC isoforms expressed by NF1 Schwann cells may result in higher basal levels of cAMP than in normal human adult Schwann cells.

Table 1. Adenylyl cyclase isoform expression in NF Schwann cells, non-NF Schwann cells, and normal human Schwann cells.

AC Isoforms	Normal Human		NF1 cells	Non-NF1
ACI	-	-	-	-
ACII	-	-	+	-
ACIII	+	+	+	+
ACIV	+	+	+	+
ACV	-	-	+	-
ACVI	-	-	-	+
ACVII	+/-	+	+	-
ACVIII	-	+	-	-
ACIX	+	+	+	-

Prostaglandin E₂ secretion in NF1 and non-NF1 Schwann cell lines

We postulated that the high levels of cAMP in Schwann cells could be sustained by PGE₂ secretion by the Schwann cells themselves. In many cell types, PGE₂ stimulates a specific adenylyl cyclase linked receptor leading to increased intracellular cAMP. To determine the levels of PGE₂ secreted from NF1 cells, serum free media from 300,000 cells was conditioned for 3 hours and were analyzed by ELISA. The results show that the 2 NF1 cell lines, T265 and ST88, secreted the most PGE₂ with 250pg/ml/mg (**figure 28**). Neonatal rat Schwann cells and normal human adult Schwann cells produced the least amount of PGE₂ with 90pg/ml/mg. The non-NF1 cell line secreted less PGE₂ than the 2 NF1 cell lines. Interestingly, the 90-8 and 88-3 cell lines secreted at least 5fold more PGE₂ than that of the ST88 and T265 levels (data not shown). Overall these results show that NF1 cells secrete the highest levels of PGE₂. The levels of PGE₂ secretion do not correlate with the levels of intracellular cAMP.

Expression of cPLA₂ and Cox-2 in Schwann cells

We wanted to determine if Schwann cells express enzymes relating to the secretion of PGE₂, such as cPLA₂ and Cox-2. To investigate expressions of cPLA₂ and Cox-2 receptor in Schwann cells, 25ug of cell lysates were analyzed by immunoblotting using cPLA₂ and Cox-2 antibodies. The immunoblotting analysis revealed that NF1 Schwann cells expressed elevated levels of cPLA₂ compared to normal human adult Schwann cells (**Figure 29A**). Similarly, levels of Cox-2 expression were much higher in NF1 cells than in normal Schwann cells as illustrated in **figure 29B**. Overall, the increased expression of cPLA₂ and Cox-2 in NF1 Schwann cells correlate with high levels of prostaglandin secretion.

Prostaglandin secretion by NF1 tumors

We established the secretion of PGE₂ by NF1 Schwann cells *in vitro*, and we wanted to extend our findings *in vivo* by analyzing levels of PGE₂ in fresh NF1 tumors. To determine the levels of PGE₂ in fresh NF1 tumors, serum free media was conditioned with tissue samples for 12 hours and levels of PGE₂ were normalized to the volume and weight of each sample. The analysis of the conditioned media revealed that 2 tumors expressed 6 and 5 pg/ml/mg of PGE₂ respectively, while 2 other tumors secreted only 1 and 1.5 pg/ml/mg respectively (**figure 30**). Controls obtained from conditioned media of human peripheral nerve had 1 pg/ml/mg. Levels of PGE₂ in NF1 tumors are somewhat variable; 2 tumors have higher PGE₂ levels than control while 2 NF1 tumors have similar levels as control. Overall, the results suggest that cells present in NF1 tumors secrete PGE₂.

Determination of prostaglandin receptor subtypes in Schwann cells

PGE₂ affects cells through a receptor mediated mechanism. PGE₂ binds preferably to the E class prostanoid (EP) receptor family, which has 4 isoforms: EP1, EP2, EP3, and EP4. (Coleman et al., 1994). To determine the EP receptors expressed by NF1, non-NF1, and normal human adult Schwann cells, RT/PCR reactions were performed using specific primers for each of the 4 receptors. PCR analysis revealed that all NF1 cells expressed both EP2 (602bp) and EP4 (553bp) receptor mRNA (**figure 31A**). However, non-NF1 Schwann cells expressed only EP2 receptor mRNA (**figure 31B**) and normal human adult Schwann cells have only the EP4 receptor mRNA (**figure 31C**). Overall, NF1 Schwann cells express a different prostaglandin receptor profile from normal and non-NF1

Schwann cells. Interestingly, EP2 and EP4 receptors are both AC coupled so that their activation can lead to increases in cAMP metabolism (Coleman et al., 1994).

Effects of PGE₂ on intracellular cAMP levels in Schwann cells

To determine the increase of cAMP levels in NF1 cells as a function of PGE₂ concentration, cells were cultured in serum free medium with increasing concentrations of PGE₂ (0.01 to 10uM) in the presence of phosphodiesterase inhibitor (200uM IBMX). After 20 minutes, cell lysates were analyzed by ELISA to determine cAMP levels. Levels of cAMP were normalized with control levels and are reported as fold increase over controls. Within 20 minutes, cells cultured with 0.01 uM of PGE₂ increased their cAMP synthesis 100-fold relative to basal levels within 20 minutes (**figure 32A**). At concentrations of 1uM or greater, the increase in cAMP levels reached a maximum of 180 fold. These results show that the NF1 cells respond to PGE₂ by increasing intracellular cAMP.

Next, using a fixed concentration of PGE₂ (1uM), we compared the increase of cAMP levels in NF1 Schwann cells to that of normal human adult Schwann cells and non-NF1 Schwann cells (**figure 32B**). Cells were cultured in serum free medium with 1uM PGE₂ and 200uM IBMX. After 20 minutes, cell lysates were analyzed by ELISA to determine cAMP levels. Levels of cAMP were normalized with control levels and are reported as fold increase over controls. The results showed that 1uM of PGE₂ induces cAMP a 40 fold increase in normal human adult Schwann cells and a 60 fold increase in non-NF1 Schwann cells (**figure 32B**). These increases are much smaller than the 200 fold increase observed with the NF1 Schwann cells. These results show that PGE₂ induce a larger increase in cAMP response in NF1 Schwann cells than in normal human adult Schwann cells and non-NF1 Schwann cells.

Effects of PGE₂ on NF1 cell proliferation

Previous studies have shown that increased levels of cAMP induce Schwann cell proliferation. We wanted to determine if increased cAMP levels stimulated by PGE₂ would increase NF1 Schwann cell proliferation. Previously, we had already determined to which extent PGE₂ increases cAMP in Schwann cells. To investigate the effects of PGE₂ on the proliferation of Schwann cells, cells were cultured in serum free medium with increasing concentrations PGE₂ (0.01 to 10uM) for 72 hours. The proliferation assay showed that PGE₂ increases in cell proliferation of NF1 Schwann cells (**figure 33**). Overall, increased cAMP levels with PGE₂ induced the

proliferation of NF1 Schwann cells. Interestingly, the PGE₂ did not potentiate the proliferation of NF1 Schwann cells cultured with PDGF BB.

PDGF BB induced cell proliferation and cox inhibitors

We have shown that the T265 NF1 cell line secretes high levels of PGE₂ and decided to determine if the secreted PGE₂ mediates NF1 cell proliferation. It is possible that the use of cyclooxygenase inhibitors (indomethacin and NS398) would decrease the production of PGE₂ and decrease NF1 Schwann cell proliferation induced by PDGF BB.

For the analysis of the production of PGE₂, serum free media from 300,000 cells was conditioned for 3 days with or without indomethacin (50uM). After 72 hours the media were analyzed by ELISA for the presence of PGE₂, and the values were normalized to protein levels. The results show that 50 uM of indomethacin reduces prostaglandin secretion of approximately 40% compared to control (figure 34A). Similar to indomethacin, we also found that 50uM of NS-398 decreases prostaglandin secretion by 40% as shown in figure 34B. Thus both inhibitors have similar effects.

For the proliferation assay, NF1 cells preincubated with NS-398 or indomethacin for 12 hours, then were cultured in serum free medium with 20ng/ml of PDGF BB alone or in combination with increasing concentrations of inhibitors for 72 hours. As shown in figure 35A, these results showed that indomethacin significantly decreased NF1 cell proliferation, but the inhibitor itself was not toxic to the cells. The decrease in cell number was dose dependent, with significant inhibition with 50uM and 100uM of indomethacin compared to control (figure 35A). Indomethacin does not discriminate between Cox-1 and Cox-2. To determine the specific cyclooxygenase enzyme contributing to the NF1 cell proliferation, NS398, a specific inhibitor of Cox-2, was used in the proliferation of NF1 cells induced by PDGF BB (figure 35B). Similarly to indomethacin, NS-398 decreased the proliferation of NF1 cells in a dose dependent manner (figure 35A). We concluded that NF1 cell proliferation is mediated by Cox-2. However, the inhibition is not complete, since other signaling pathways are involved in the proliferation of NF1 cells.

PDGF BB induced cell proliferation and PKA inhibitor

Previously, we have shown that NF1 cells have elevated cAMP levels, which may contribute to the aberrant proliferation of NF1 cells. Since the downstream effector of cAMP is PKA, we evaluated the extent to which cAMP mediates NF1 cell proliferation induced by PDGF BB by using the PKA inhibitor H89. To investigate the effect of H89 on NF cell proliferation, NF1 cells were cultured in serum free medium with 20ng/ml of PDGF BB in combination with increasing concentration of the H89 inhibitor for 72 hours. As shown in figure 36, the PKA inhibitor H89 decreased NF1 cell proliferation in a dose dependent manner. This data is consistent with the viewpoint that NF1 cell proliferation induced by growth factor, such as PDGF BB, is mediated by PKA.

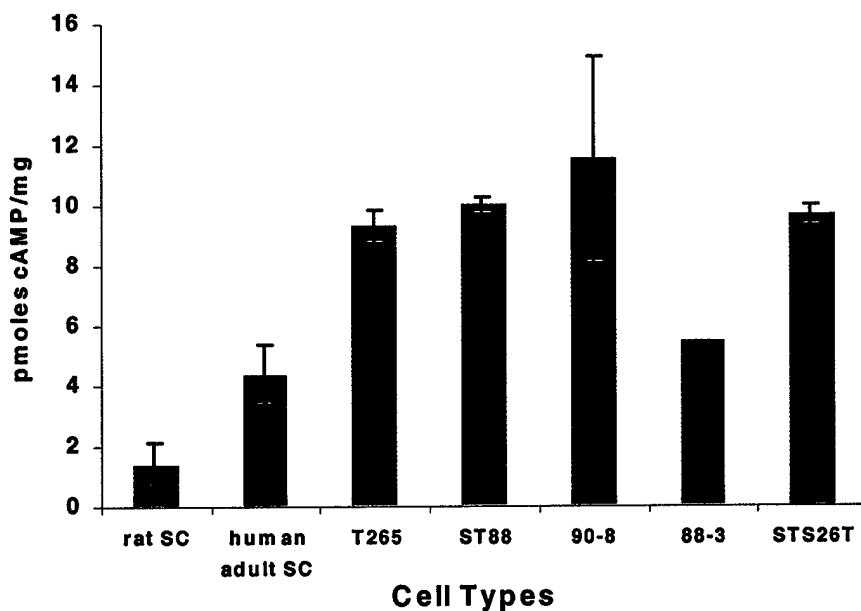


Figure 26. NF1 Schwann cells have higher basal cAMP levels than normal Schwann cells. Schwann cells were incubated in serum free medium with the phosphodiesterase inhibitor, IBMX (200uM), for 30 minutes. Cells were lysed and concentrations of cAMP were determined by ELISA in duplicate. Values are mean of 3 different experiments. Error bars represent +/- standard error deviation.

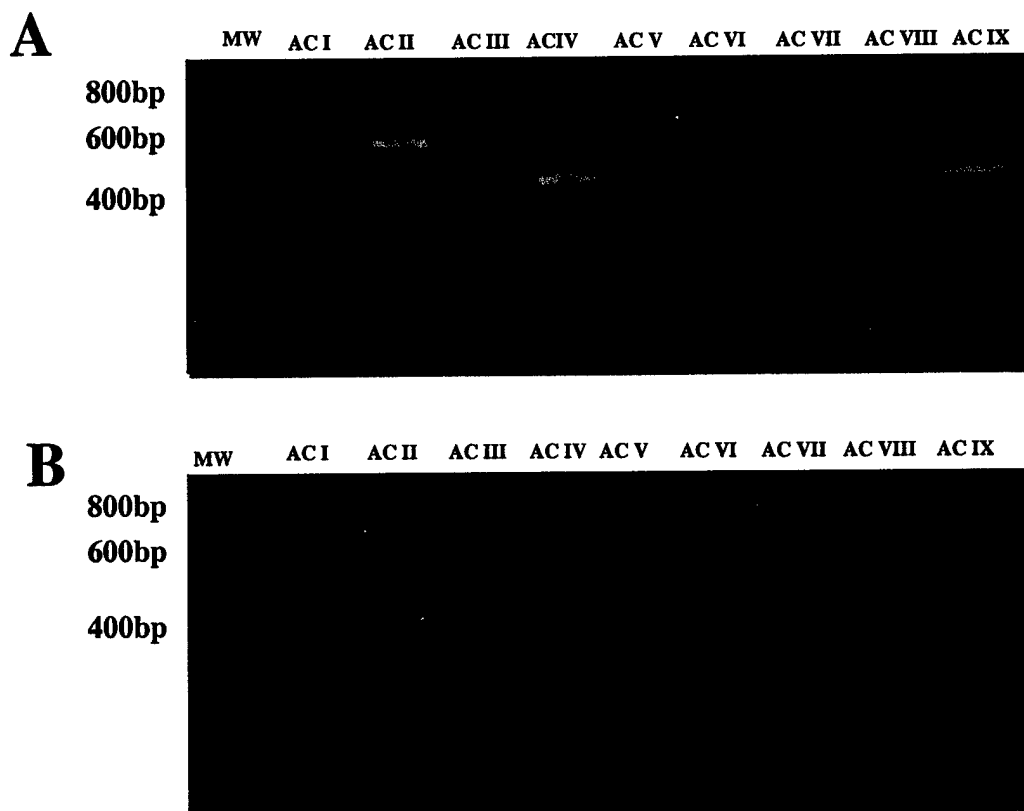


Figure 27. Types of adenylyl cyclases mRNA expression in Schwann cells. RT/PCR amplification products for AC I (1044 bp), AC II (581 bp), AC III (772bp), AC IV(559bp) AC V(300bp), AC VI (774), AC VII (772 bp), AC VIII (695bp), and AC IX (469bp) in the T265 Schwann cell line (A) and in normal human adult Schwann cells (B). PCR products were separated on a 2% agarose gel stained with ethidium bromide. Molecular weight markers (MW) are derived from 100 base pairs ladder. These photographs are a representative experiment repeated twice.

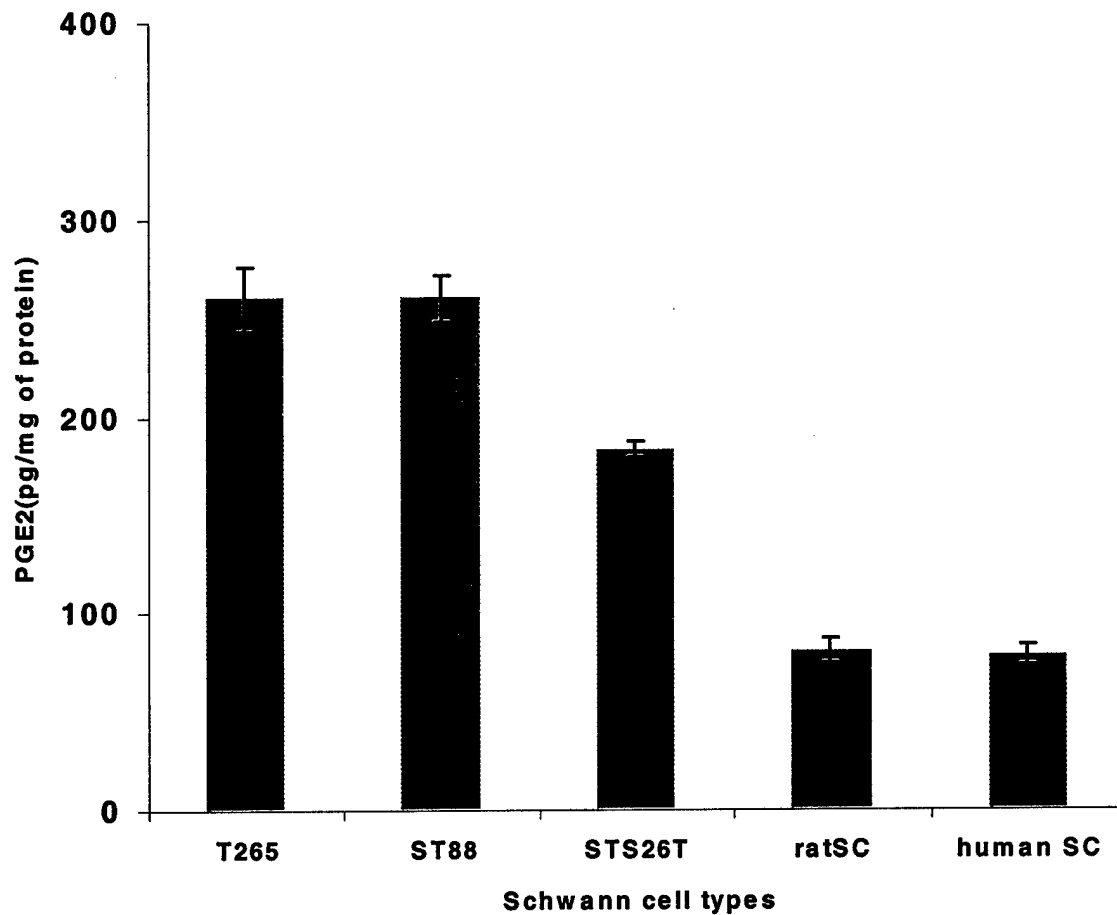


Figure 28. NF1 Schwann cells secrete higher levels of PGE2 than normal and non-NF1 Schwann cells. Cells were incubated for 3 hours in serum free medium. Conditioned media were collected and concentrations of PGE2 were measured by ELISA in triplicate. Values are expressed as mean 3 different experiments. Error bars represent +/-standard error of the mean.

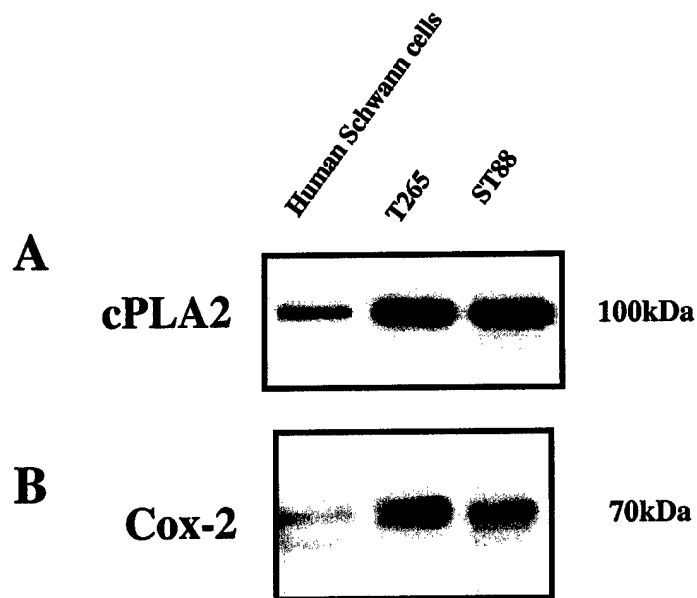


Figure 29. Expression of cPLA₂ and Cox-2 in Schwann cells. Schwann cells were lysed and 25ug of proteins were analyzed by immunoblotting with an antibody specific for cPLA₂ (A) or Cox-2 (B). These immunoblots are a representative experiment repeated 3 times with similar results.

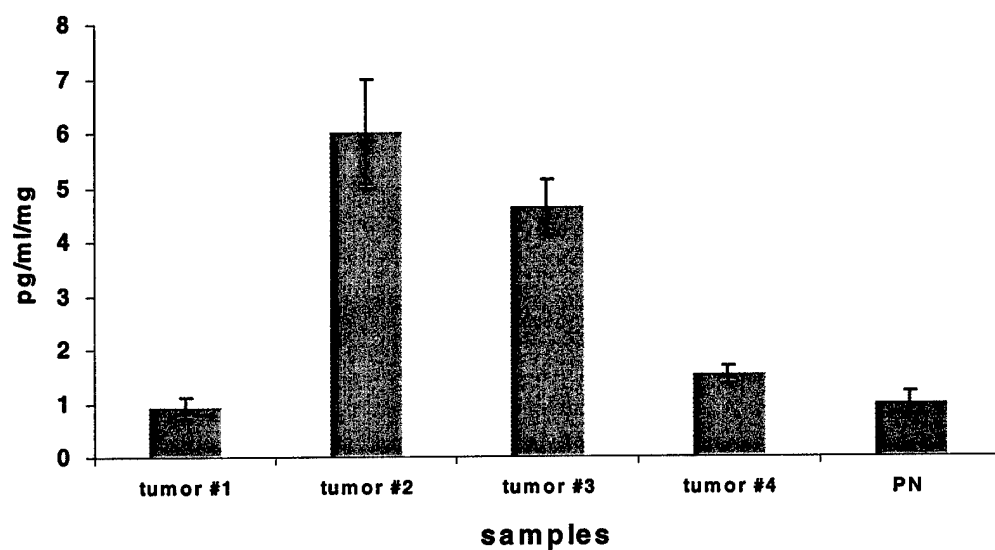


Figure 30. PGE₂ secretion by NF1 tumors. Tissue samples were incubated in serum free media overnight. Conditioned media were analyzed by ELISA in triplicate. Values are expressed as PGE₂ secretion levels that were normalized to protein levels. Error bars represent +/- standard deviation.

PLATELET-DERIVED GROWTH FACTOR BB STIMULATES
NEUROFIBROSARCOMA-DERIVED SCHWANN-CELL-DIRECTED MIGRATION

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Short Running Title: PDGF BB Induces NF1-Cell Migration

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ABSTRACT

Malignant peripheral-nerve tumors or neurofibrosarcomas are the major type of malignant tumors present in Neurofibromatosis type 1 (NF1) patients. Mutations in the *nf1* gene, which codes for a putative tumor suppressor, result in increased Ras activity in neurofibrosarcoma-derived Schwann cells and Schwann-cell hyperplasia.

Neurofibrosarcoma-derived cells also display aberrant expression of specific growth-factor receptors, such as platelet-derived growth-factor (PDGF) receptors or Kit tyrosine kinase receptor. It has been established that PDGF BB is a potent mitogen for neurofibrosarcoma-derived Schwann cells. This research indicates that PDGF BB strongly stimulates the migration of neurofibrosarcoma-derived Schwann cells in a Boyden-like chamber assay. The effect of PDGF BB is not simply chemokinetic, but chemotactic since it was largely dependent on the presence of a gradient of PDGF BB. PDGF AA, fibroblast growth factor 2 or stem-cell factor (Kit ligand), previously shown to be mitogens for neurofibrosarcoma cells, are poor chemoattractants. PDGF BB also stimulates neurofibrosarcoma-derived cell invasion of a reconstituted matrix. NF1-derived cells secrete increased levels of matrix metalloproteinases 2 and 9 relative to normal Schwann cells, which could explain their high invasive potential. Results of this study indicate that PDGF BB, through its mitogenic and chemotactic properties, could contribute to the malignant phenotype of neurofibrosarcoma-derived Schwann cells.

Key words: NF1, PDGF BB, migration, Schwann cells, MMP

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a genetic disease, with a variety of clinical manifestations including café-au-lait spots, hamartomas of the iris, learning disabilities and neurofibromas (Riccardi 1991). Neurofibromas are benign tumors of the peripheral nerve that can become malignant and form neurofibrosarcomas or malignant peripheral-nerve-sheath tumors (MPNST). These tumors are composed principally of Schwann-like cells. Even though a mutation in a tumor suppressor gene coding for a protein called neurofibromin has been identified in NF1 patients (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990, Wallace *et al.*, 1990), the mechanism of malignant transformation remains unclear. Neurofibromin belongs to the family of Ras-GTPase-activating proteins (Ras-GAPs) and is a negative regulator of the Ras pathway (Basu *et al.*, 1992; DeClue *et al.*, 1992). Mutations in the *nf1*-gene neurofibromin result in increased levels of activated (GTP-bound) Ras (Kim *et al.*, 1995; Guha *et al.*, 1996). However, Schwann cells from *nf-1* knock-out mice, which display increased Ras activity, do not show increased proliferation (Kim *et al.*, 1995). These neurofibromin-deficient cells also displayed invasive properties independent of Ras activity (Kim *et al.*, 1997). These observations suggest that increased Ras activity alone cannot explain the NF1-Schwann-cell-transformed phenotype and that genetic or epigenetic events subsequent to NF1 mutation are required for transformation. For instance, inactivating mutations at the p53 gene cooperate with loss of *nf1* for malignant transformation in mouse-tumor models for NF1 (Cichowski *et al.*, 1999; Vogel *et al.*, 1999). Over expression of the angiogenic factor midkine was also proposed to contribute to neurofibroma growth (Mashour *et al.*, 2001).

It has previously been shown that Schwann-cell lines derived from neurofibrosarcomas contain abnormally high levels of growth-factor receptors, such as the platelet-derived growth-factor (PDGF) receptors and the tyrosine kinase receptor, Kit (Ryan *et al.*, 1994; Badache *et al.*, 1998; Badache et DeVries, 1998). Accordingly, PDGF BB was shown to be a potent mitogen for neurofibrosarcoma-derived Schwann cells, indicating that increased expression of PDGF receptors could contribute to Schwann-cell hyperplasia in NF1. Kit ligand (also called stem-cell factor, SCF), PDGF AA and fibroblast growth factor (FGF) 2 induced a more modest mitogenic response in neurofibrosarcoma-derived cells (Badache and DeVries, 1998). Immunoreactivity with c-Met, the receptor for the hepatocyte growth factor (HGF), was found to be strikingly greater in MPNSTs in contrast to benign tumors (Rao *et al.*, 1997). Therefore, aberrant expression of growth-factor receptors could contribute to the progression of Schwann cells toward a transformed phenotype.

The most life-threatening aspect of malignant cells lies in their metastatic ability. Metastasis formation involves a series of steps including the attachment of the tumor cells to the extracellular matrix, degradation of the matrix component by proteolytic enzymes and migration through the damaged matrix to the target tissue (Stetler-Stevenson *et al.*, 1993). A variety of agents appear to stimulate the motile response of tumor cells including growth factors and components of the extracellular matrix. Therefore, investigation of the possible influence of growth factors such as PDGF AA, PDGF BB, FGF2 or SCF, that are mitogenic for neurofibrosarcoma-derived cells, on neurofibrosarcoma-cells motility and invasiveness.

The observations reported herein show that, among the growth factors tested,

only PDGF BB can induce migration of neurofibrosarcoma-derived Schwann cells and invasion of a reconstituted extracellular matrix. Neurofibrosarcoma-derived Schwann cells were also found to secrete increased levels of matrix metalloproteinases (MMP), specifically MMP2 and MMP9, relative to normal Schwann cells. These results indicate that PDGF BB might play an important role in the invasive properties of the transformed Schwann cells present in the NF1 tumors.

MATERIAL AND METHODS

Cells and reagents

The T265-2c cell line (developed by Karen Klein in our laboratory) and the ST88-14 cell line (obtained from Jonathan Fletcher, Brigham and Women Hospital, Boston, MA) were derived from malignant peripheral-nerve tumors from patients with NF1. The STS-26T cells (obtained from William Dahlberg, Harvard School of Public Health, Boston, MA) were derived from an isolated grade III malignant schwannoma in an individual without NF1. The RSC-96 line is a spontaneously-transformed rat Schwann-cell line. Primary rat Schwann cells were obtained as described previously (Neuberger and DeVries, 1993). Cells were maintained in Dulbeccos's modified Eagle medium (DMEM) supplemented with 10 % fetal calf serum (FCS, Hyclone, Logan, UT). Recombinant human PDGF AA, PDGF BB, FGF2 and SCF were obtained from R&D Systems (Minneapolis, MN).

Immunoprecipitation and Western blotting

Cells were lysed in RIPA buffer (1% Triton X-100, 0.5% sodium dodecylsulfate and 0.5% sodium deoxycholate in phosphate-buffer saline containing 1 mM sodium orthovanadate and protease inhibitors). Cell extracts were incubated in the presence of an agarose-coupled phosphotyrosine antibody (Santa-Cruz Biotechnology, Santa-Cruz, CA) at 4°C for 2 hours. After several washes, immunoprecipitated proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Dupont NEN, Boston, MA). Non-specific binding sites were blocked with 1% gelatin and the membrane probed with specific primary antibodies (anti-PDGF receptor, anti-PLC (phospholipase C), anti-p85 or anti-Grb2 from Santa-Cruz Biotechnology). After several washes in phosphate-buffer saline (PBS) containing 1% Tween-20, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) and developed using enhanced chemiluminescence (Dupont-NEN).

Migration and invasion assays

Migration assays were performed in 8 μ m-pore-size Transwell dishes (Costar Corp., Cambridge, MA) and colorimetric measurement of cell migration was carried out as described by Muir *et al.* (1993). Cells (5×10^4), harvested in PBS/EDTA, were loaded on the polycarbonate filters of the Transwell units in 100 μ l serum-free medium, while growth-factor-containing serum-free medium was added to the bottom chamber. When

indicated, PDGF BB was added to both chambers. Cells were allowed to migrate overnight at 37°C. Non-migrated cells were wiped from the filter with a cotton swab and the migrated cells were fixed in 4% buffered formaldehyde. Filters were stained with 1% toluidine blue in 1% borax, rinsed with water and allowed to air dry. After photography under an inverted microscope, filters were placed in 200 l of 1% SDS and the absorbance of the solubilized dye was measured at 595 nm. For invasion assays, filters were first soaked with a solution of 5% Matrigel (Collaborative Biomedical Products, Bedford, MA) in cold H₂O for 2 hours. The Matrigel solution was air-dried overnight. Cells were loaded on the coated filters and allowed to migrate for 48 hours. The number of cells that invaded Matrigel and migrated through the filters was evaluated as described above. Assays were performed in triplicate wells in two to three separate experiments. In parallel experiments, for construction of a standard curve, cells were plated in 96-well plates (n=5), then fixed and stained with 1% toluidine blue as described above.

Zymographic analysis

Nearly confluent cultures were grown in serum-free medium for 48 hours. The medium was collected, clarified by centrifugation and concentrated approximately 20 times. Equivalent amounts of protein were electrophoresed in non-reducing conditions in a 10% polyacrylamide solution containing 1.5 % gelatin. The gel was washed in 2.5 % Triton X-100 in 50 mM Tris-HCl (pH 7.5) to eliminate the SDS and incubated at 37°C in 0.15M sodium chloride, 10 mM calcium chloride, 50 mM Tris-HCl (pH 7.5) for 16 hours. The gel was stained with Coomassie Brilliant Blue and briefly destained.

RESULTS

PDGF BB activates multiple intracellular pathways in T265-2c cells

Badache and DeVries (1998) showed that PDGF BB, which is capable of binding both PDGF receptor α and β strongly stimulated the tyrosine phosphorylation of PDGF receptor β in neurofibrosarcoma-derived Schwann cells. The recruitment to the PDGF receptors of adaptor molecules or enzymes involved in major signaling pathways was analyzed to study the intracellular pathways activated by PDGF BB in these cells. T265-2c cells, cultured in serum-free medium in the presence of 50 ng/ml PDGF AA or PDGF BB, were lysed and subjected to immunoprecipitation using an anti-phosphotyrosine antibody. Immunoprecipitated proteins were analyzed by Western blotting, using antibodies specific for the PDGF receptor β , ν phospholipase C ν (PLC ν the p85 subunit of phosphatidylinositol-3 kinase (PI3K) or the adaptor molecule Grb2 (Fig.1). PDGF BB induced the formation of a tyrosine phosphorylated complex containing PLC ν PI3K and Grb2 in association with the PDGF receptor β . Direct immunoprecipitation of the respective molecules, followed by phosphotyrosine immunodetection, showed that PLC ν was specifically tyrosine phosphorylated upon PDGF BB stimulation, whereas tyrosine phosphorylation of p85 and Grb2 was not observed (results not shown). PDGF AA, which is known to specifically interact with PDGF receptor β , did not induce the recruitment of activated signaling molecules, indicating that PDGF BB-induced activation of these major signaling pathways occurs through PDGF receptor β .

PDGF BB induces migration of T265-2c cells

Chemotactic activity of PDGF BB for the neurofibrosarcoma-derived Schwann-cell line was evaluated in Boyden-type units using a colorimetric assay. Cells were loaded in the upper chamber and allowed to migrate for 16 hrs. Cells that did not migrate were eliminated, while cells that migrated to the bottom of the polycarbonate membrane were stained with toluidine blue. Microscopic observation showed that virtually no cells migrated through the membrane when the lower chamber contained only serum-free medium, indicating that cells are unable to migrate through the 8 μ m pore membrane by passive diffusion or in response to an autocrine stimulation (Fig. 2A, control). In contrast, addition of PDGF BB to the bottom chamber induced cell migration toward the lower compartment. The number of migrating cells increased with increasing amount of PDGF BB in the lower chamber (Fig. 2A).

To quantify the extent of PDGF BB-induced migration, toluidine-blue stained cells were solubilized in 1 % SDS and absorbance measured spectrophotometrically, the conditions used in this assay were verified to ensure that there was a linear relationship between cell number and absorbance (correlation coefficient: 0.998, between 1,250 and 50,000 cells, data not shown). The response of T265-2c cells to increasing amounts of PDGF BB was then evaluated (Fig. 2B). PDGF BB induced the neurofibrosarcoma-derived cells to migrate in a dose-dependent manner. Effects on migration were visible from 1 ng/ml PDGF BB with a maximum effect between 25 and 50 ng/ml. Migration was reduced at higher doses.

It has previously been shown that PDGF BB is a potent mitogen for neurofibrosarcoma-derived Schwann cells (Badache and DeVries, 1998). However,

increased cell proliferation cannot account for the increased number of cells migrating in the presence of PDGF BB. Indeed, when total cell number was evaluated in parallel cultures grown in the experimental conditions used for the migration assay, the proliferative response to PDGF BB was different from the chemotactic response both quantitatively and qualitatively. An increase in cell number was observed only at doses higher than 10 ng/ml PDGF BB and was limited to 1.5 fold at 100 ng/ml PDGF BB (data not shown).

PDGF BB-induced migration: chemotaxis versus chemokinesis

In order to test whether PDGF BB influences T265-2c cell migration by inducing increased random motility of the cells (chemokinesis) or by stimulating directed migration along the PDGF BB gradient (chemotaxis), cells were assayed for migration in the presence of PDGF BB in only the lower compartment of the Boyden unit (as described before) or with equal concentrations of PDGF BB in the upper and lower compartments (Fig. 3). When PDGF BB was added to the lower compartment only, the expected dose-dependent increase in cell migration was observed. However, in the absence of a PDGF BB gradient, cell migration was abolished (Fig. 3). This result indicates that the effect of PDGF BB on migration of neurofibrosarcoma-derived cells is mainly chemotactic.

Effect of other growth factors on NF1-cell migration.

These results demonstrate that PDGF BB, which was previously shown to be a

potent mitogen for neurofibrosarcoma-derived Schwann cells, is also a strong chemoattractant for these cells. Since FGF2, PDGF AA and SCF are also, to a lesser extent, mitogens for neurofibrosarcoma-derived Schwann cells, the effect of these factors on T265-2c migration was evaluated (Fig. 4). The three factors tested had a very limited effect on T265-2c cell migration. Therefore, among the known mitogens for neurofibrosarcoma-derived cells, only PDGF BB is also a potent chemoattractant.

Effect of PDGF BB on invasion of a basement membrane.

Tumor-cell invasion is a complex process involving sequential steps, including the ability of tumor cells to degrade basement membrane components and to invade tissues. Therefore, the ability of T265-2c cells to migrate through a reconstituted basement membrane was evaluated. The invasive ability of T265-2c cells was evaluated in the presence of increasing concentration of PDGF BB. PDGF BB induced migration of the T265-2c cells through Matrigel in a dose-dependent manner, with a maximum effect observed at 50 ng/ml PDGF BB (Fig.5A).

Basement membrane degradation by metastatic cells involves various degrading enzymes, including metalloproteinases. The activity of type IV collagenases secreted into the cell-culture medium was measured by zymographic analysis of conditioned medium from neurofibrosarcoma-derived cell lines, from a cell line derived from a sporadic schwannoma (from a non-NF1 patient), from an immortalized-rat-cell line and from primary rat Schwann cells (Fig. 6). The two neurofibrosarcoma-derived Schwann-cell lines secreted readily detectable amounts of MW 72,000 type IV collagenase (MMP-2 or gelatinase A) and MW 92,000 type IV collagenase (MMP-9 or gelatinase B). The

schwannoma-derived cells secreted high levels of MMP2 only, while the immortalized-rat-cell line secreted both low levels of MMP-2 and high levels of MMP-9. Primary rat Schwann cells (SC) secreted low levels of MMP-2 only.

To evaluate the effect of PDGF BB on the expression and secretion of the metalloproteinases, T265-2c cells were treated with increasing amounts of PDGF BB and the conditioned medium was analyzed for gelatinolytic activity. PDGF BB had a limited effect on MMP activity, inducing a modest increase in the amount secreted MMP9 and a small decrease in MMP2 secretion. The ratio of MMP2 to MMP9 was decreased in a dose-dependent manner from a ratio of two in the absence of PDGF BB to a ratio of one in the presence of 50 ng/ml PDGF BB (results not shown).

DISCUSSION

It has been previously shown that PDGF BB could contribute to Schwann-cell transformation in NF1, because of its potent mitogenic effect on neurofibrosarcoma-derived Schwann cells (Badache and DeVries, 1998). The results of this investigation indicate that PDGF BB can also contribute to other crucial steps in Schwann-cell progression toward a malignant phenotype by stimulating migration of neurofibrosarcoma-derived Schwann cells and invasion through a reconstituted basement membrane.

Several studies of the intracellular pathways induced by PDGF BB have been carried out in smooth muscle cells or fibroblasts, with contradictory results. Ras, mitogen activated protein kinase (MAPK), Rac, PI3K or PLC γ /Ca $^{++}$ have all been implicated in the migration of these cells (Kundra *et al.*, 1994; Wennstrom *et al.*, 1994;

Kundra *et al.*, 1995 Higaki *et al.*, 1996; Hinton *et al.*, 1998). However, equivalent studies have not been carried out in malignant cells. Ras activity was shown to be necessary for neurofibrosarcoma-derived Schwann-cell proliferation (Basu *et al.*, 1992), whereas PI3K mediates PDGF BB-induced proliferation (I. Dang and G. H. DeVries, unpublished results). The fact that these cells have high basal levels of activated Ras, but are unable to migrate in the absence of PDGF BB indicates that activation of the Ras/MAPK pathway is not sufficient for neurofibrosarcoma-derived-Schwann-cell migration. Preliminary results using specific pharmacological inhibitors indicate that the Ras/MAPK pathway, the PI3K pathway and PLC γ are required for the full migratory response to PDGF BB (Badache and DeVries, unpublished results). PDGF AA, FGF2 and SCF, which are capable of activating the same signaling pathways, failed to stimulate migration of the neurofibrosarcoma-derived cells, probably because they do not activate these pathways above a certain threshold required for cell migration.

The malignant potential of cancer cells is dependent not only on tumor development but also on the subsequent metastatic behavior (Stetler-Stevenson *et al.*, 1993). Numerous studies have established a link between abnormal expression of PDGF receptors in cells from malignant gliomas, osteosarcomas or melanomas and uncontrolled cell proliferation and tumor development. However, while the role of PDGF BB in cell migration has been extensively studied in development, wound healing, and pathologies such as atherosclerosis, few studies have investigated the potential contribution of PDGF BB to migration of metastatic cells (Allam *et al.*, 1992; Matsui *et al.*, 1993; Wach *et al.*, 1996; Klominek *et al.*, 1998). The data reported here show that PDGF BB is not only a potent mitogen, but also a potent chemoattractant for

neurofibrosarcoma-derived Schwann cells and might play a critical role in the progression of Schwann cells toward malignancy in NF1. In the absence of PDGF BB, very little or no migration of neurofibrosarcoma-derived Schwann cells was observed, while doses of PDGF BB as low as 1 ng/ml could significantly stimulate neurofibrosarcoma-derived cell motility. The absence of basal migration, as well as the absence of activated PDGF receptor in unstimulated cells, argues against the existence of a PDGF/PDGF receptor autocrine motility loop in neurofibrosarcoma-derived cells. This is in contrast to osteosarcoma cells or malignant mesothelioma cells which co-express PDGF receptors and PDGF BB (Allam *et al.*, 1992; Klominek *et al.*, 1998). The absence of PDGF secretion by neurofibrosarcoma-derived cells is in accordance with the observation that PDGF BB functions as a chemotactic factor. Indeed, autocrine motility factors secreted by tumor cells generate high local concentrations of this factor which can stimulate random migration, while chemotactic substances require the formation of a gradient in order to attract metastasizing tumors to specific locations (Liotta *et al.*, 1986). Even though growth factors such as PDGF AA, FGF2 or SCF had very little effect on cell motility, it is possible that other factors may influence the motility of neurofibrosarcoma-derived cells. It would be of particular interest to evaluate the effect of HGF on these cells. HGF has been shown to promote cell motility and invasion on a variety of carcinomas and sarcomas (Jeffers *et al.*, 1996). The fact that expression of the receptor for HGF is increased in malignant peripheral-nerve tumors (Rao *et al.*, 1997) suggests that HGF could be a motility factor for neurofibrosarcoma-derived cells.

PDGF BB induced neurofibrosarcoma-derived Schwann-cell motility, and also their ability to invade a reconstituted basement membrane. A direct correlation has been

observed between the production of MMPs by tumor cells and their metastatic behavior (Matsui *et al.* 1993; Curran *et al.*, 1999). Activation of tyrosine kinase receptor such as ErbB2, the epidermal growth-factor receptor or colony-stimulating factor 1 receptor was shown to up-regulate MMP activity (Sapi *et al.*, 1996; Tan *et al.*, 1997; Kondapaka *et al.*, 1997). However, activation of PDGF receptor by PDGF BB had only a modest effect on the levels of secreted MMPs and therefore the effect of PDGF BB on neurofibrosarcoma-derived Schwann-cell invasion is not due to increased levels of MMPs. Actually, the neurofibrosarcoma-derived cell lines displayed high basal levels of secreted MMP2 and MMP9, relative to normal Schwann cells. Two benign neurofibroma-derived Schwann cells were previously shown to express increased levels of MMP2, and also MMP9 for the most invasive, relative to control cells (Muir, 1995). Thus, over expression of MMPs may be an early event in the process leading to NF1 Schwann-cell transformation. Interestingly, transfection of human ovarian cancer cells by activated Ras was shown to increase MMP9 activity (Gum *et al.*, 1996). It is, therefore, tempting to speculate that increased basal Ras activity in neurofibromin-deficient Schwann cells (DeClue *et al.*, 1992; Guha *et al.*, 1996) could be responsible for increased MMP9 and possibly MMP2 activity. Because of the already high basal levels of MMP activity, neurofibrosarcoma-derived Schwann cells only require stimulation of the motile response by PDGF BB to become invasive.

In conclusion, PDGF BB appears to be an important factor in the development of malignant tumors in NF1. Indeed, the data indicate that PDGF BB may not only favor the growth of the tumors, but that it could also trigger the metastatic process. Future studies, aiming at better defining the role of growth factors such as PDGF BB in the

progression of neurofibromas towards malignancy, may lead to the development of novel therapeutic approaches for NF1.

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FIGURE LEGENDS

Figure 1: PDGF BB triggers the formation of a tyrosine-phosphorylated signaling complex. T265-2c cells were stimulated with 50 ng/ml PDGF AA or PDGF BB for 10 min. The cell lysates were immunoprecipitated using a phosphotyrosine-specific antibody and immunoblotted with specific antibodies for PDGF receptor β , PLC, the p85 subunit of phosphatidylinositol-3 kinase or the Grb2 adaptor molecule.

Figure 2: PDGF BB stimulates T265-2c cell migration. **A:** T265-2c cells were seeded in the top chamber of a Boyden-like unit and allowed to migrate through a 8 μ m-pore-size polycarbonate membrane toward the bottom chamber containing the indicated amounts of PDGF BB. Migrated cells were stained with toluidine blue. Bar is 100 μ m. **B:** Migrated cells were stained with toluidine blue and the dye solubilized in 1% SDS, before colorimetric analysis.

Figure 3: PDGF BB stimulates T265-2c directed migration. Migration of T265-2c cells was evaluated as described in Fig. 2, in the presence of PDGF BB in the bottom chamber only (closed bars) or in the presence of equal amounts of PDGF BB in the top and bottom chambers (hatched bars).

Figure 4: PDGF AA, FGF2 and SCF affects T265-2c cell migration. Migration of T265-

2c cells was evaluated as in Fig. 2 in the presence of the indicated amounts of growth factor in the bottom migration chamber or in the presence of 10 ng/ml PDGF BB.

Figure 5: PDGF BB Stimulates neurofibrosarcoma-derived Schwann-cell invasion.

Invasion of a reconstituted matrix by T265-2c cells was evaluated in the presence of the indicated amounts of PDGF BB, as in Fig. 2.

Figure 6: The neurofibrosarcoma-derived Schwann-cell lines, T265-2c and ST88-14, the schwannoma-derived cell line, STS26T, the RSC-96 rat immortalized cells and primary Schwann cells (SC) were grown in serum-free medium for 48 hours. Conditioned medium was analyzed for gelatinase activity.

Figure 1

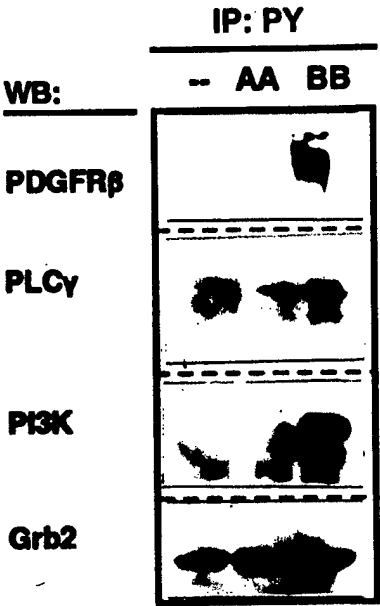


Figure 2b

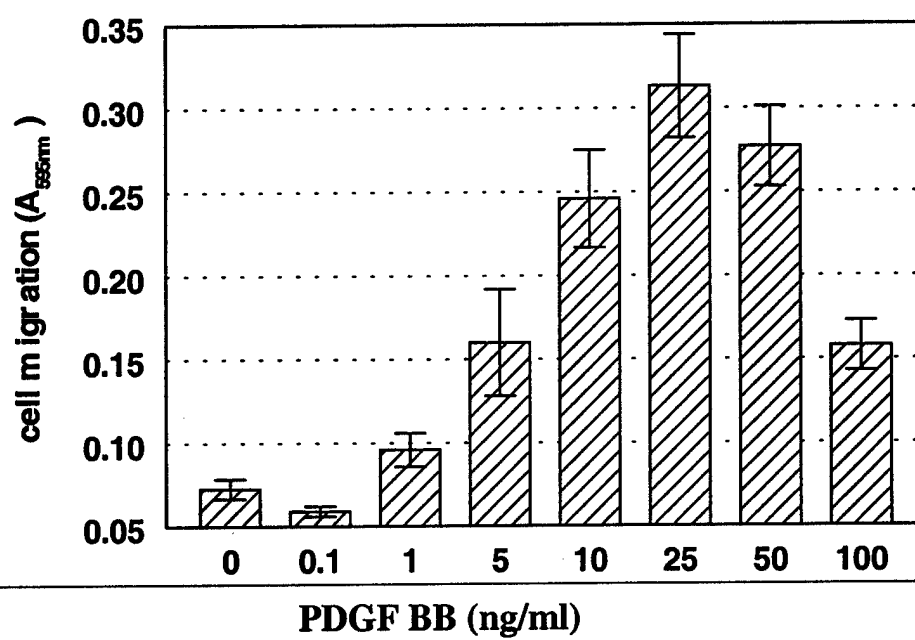


Figure 3

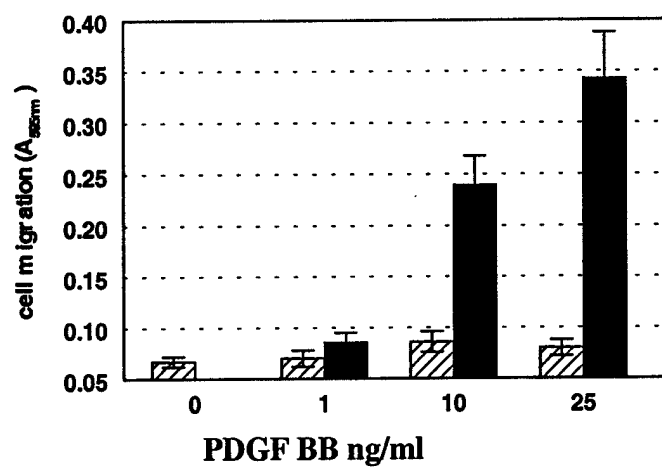


Figure 4

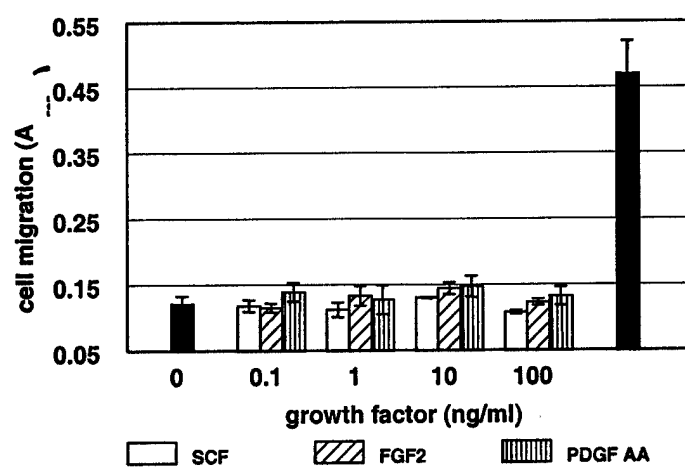


Figure 5

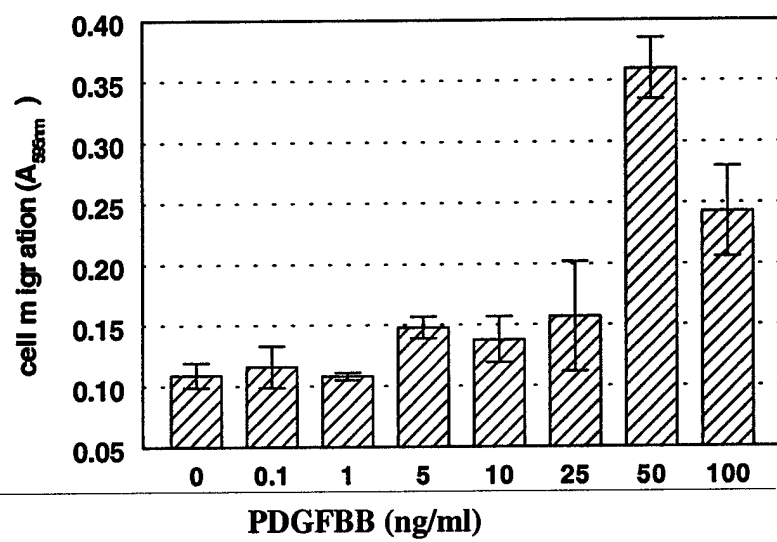
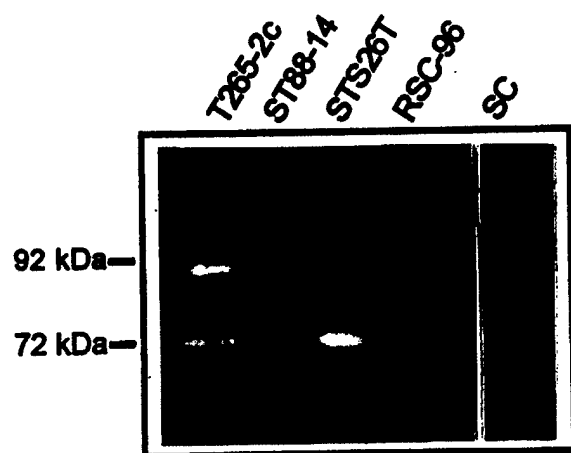


Figure 6



NF1 Schwann Cells: Normal PI3 Kinase and ERK Signal Transduction Pathways but Aberrant Calcium Metabolism in Response to PDGF BB

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Running Title: Signal Transduction in NF-1 Schwann Cells

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a genetic disease characterized by the loss of neurofibromin leading to the formation of either benign Schwann cell tumors (neurofibromas) or highly invasive and metastatic Schwann-cell tumors (neurofibrosarcomas). The growth-factor receptor expression levels and signal transduction pathways of NF1 Schwann cells and normal adult Schwann cells were compared to better understand the molecular basis of hyperplasia in NF1. The PDGF receptor levels are higher in NF1 tumors than the PDGF receptor levels found in normal adult peripheral nerves. However, the PDGF receptor levels are similar in normal adult and NF1 Schwann cells. Signal transduction downstream of the PDGF receptor was also studied in the two cell types. Erk and Akt of the PI3K pathway are phosphorylated in response to PDGF BB in both cell types. However, PDGF stimulated changes in cellular calcium levels reveal that PDGF BB increased intracellular calcium levels in NF1 Schwann cells but no change in calcium was observed in normal adult Schwann cells. The calcium response in the NF-1 Schwann cells originated in the internal compartment of the cell rather than from its extracellular compartment. The calcium downstream effector calmodulin kinase II (CAMKII) is phosphorylated in response to PDGF BB in NF-1 Schwann cells. In addition, and the growth of NF1 Schwann cells stimulated by PDGF BB is decreased with the CAMKII inhibitor, KN62. These results are consistent with the view that the aberrant activation of the calcium signaling pathway in NF-1 Schwann cells may contribute to the formation of NF1 tumors.

Keywords: NEUROFIBROMATOSIS TYPE 1, AKT, CALCIUM, CALMODULIN KINASE II (CAMKII), SCHWANN CELLS

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an inherited disease, which is characterized by a number of symptoms including neurofibromas, café-au-lait spots, and learning disabilities (Friedman *et al.*, 1999). The hallmark of NF1 is the formation of either benign Schwann-cell tumors of the peripheral nerve sheath (neurofibromas) or lethal, malignant, peripheral-nerve-sheath tumors. The tumors of this genetic disease are caused by defects of the NF1 gene resulting in the absence of its protein product, neurofibromin. Although the function of neurofibromin is not known, its loss leads to the elevated levels of the activated form of Ras (Ras-GTP). Past studies have shown that Ras-GTP levels are elevated in neurofibrosarcoma tumors from NF1 patients and Schwann cells derived from mice lacking NF1 (Feldkamp *et al.*, 1999; Kim *et al.*, 1995).) However, the link between the development of Schwann-cell tumors and the loss of neurofibromin has not yet been clearly established.

As in several types of cancer, aberrant expression levels of growth-factor receptors may play a role in cellular transformation. Schwann-cell lines derived from NF1 tumors have numerous growth-factor receptors that are aberrantly regulated. For instance, Badache *et al.* (1998 a , 1998b) reported that Schwann cells overexpress c-kit and PDGF receptors. In addition, increased EGF receptor levels have been documented on the surface of NF1 Schwann cells (DeClue *et al.*, 2000). Interestingly, NF1 Schwann cells lack Erb-B2 receptors (Badache *et al.*, 1998 a, b), which are activated in response to neuregulin, the most potent growth-factor for normal Schwann cells (Morrissey *et al.*, 1995). Taken together, these results implicate aberrant growth-factor receptor expression levels play a critical role in the development of Schwann-cell tumors in NF1 patients.

The activation of PDGF receptors in NF1 cells may lead to abnormal activation of intracellular signaling pathways contributing to increased proliferation of NF1 Schwann cells compared to normal human adult Schwann cells. In this study we found that both the PI3 kinase pathway and the calcium pathway uniquely mediate the NF1 Schwann-cell proliferation induced by PDGF BB. Further, the ERK pathway was activated in response to PDGF BB.

MATERIALS AND METHODS

Reagents and Equipment

Polyclonal anti-PDGF receptor β antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human PDGF BB was purchased from R&D Systems (Minneapolis, MN). Akt and phospho-Akt antibodies were purchased from Cell Signaling System (Boston, MA). Erk and phospho-ERK antibodies were purchased from Transduction Laboratories (San Diego, CA). KN62 was purchased from Biomol (Plymouth Meeting, PA). Recombinant neuregulin (amino-acids 14-241) was a gift from Amgen (Thousand Oaks, CA). Forskolin and LY294002 were from Calbiochem (La Jolla, CA).

Schwann cell Lines

The cell lines, ST88-14, T265, STS 26T, used in these studies were the same as those described by (Badache *et al.*, 1998 a,b). The 90-8 and 88-3 cell lines were obtained from Dr. Jeff DeClue and have been previously described (DeClue *et al.*, 1992).

Cells were cultured as monolayers in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Hyclone, Logan, UT), 100 units/ml penicillin and 100 ug/ml streptomycin, and maintained at 37°C in a humid atmosphere of 10% CO₂ and 90% air.

For proliferation assay and western blotting, cells were serum-starved overnight and then cultured in serum-free media with PDGF BB alone or in combination with inhibitors.

Normal Human Schwann Cell Preparation

Primary human Schwann-cell cultures were established as described by Casella *et al.*, (2000) (Miami Project, University of Miami, Miami, Fla). Fresh human peripheral nerves (*cauda equina*) were obtained through Dr. Wood from transplant patients.

For ten days, nerve fragments were incubated in DMEM containing 10% heat-inactivated fetal bovine serum, 2uM forskolin, 10nM neuregulin β 1, 50 U/ml penicillin and 0.05 mg/ml streptomycin. Then the fragments were dissociated in DMEM containing 10% heat-inactivated fetal bovine serum, 2uM forskolin, 10nM neuregulin β 1, 50U/ml penicillin and 0.05 mg/ml streptomycin, 0.05% collagenase and 0.25 % dispase in an Erlenmyer flask. The nerve fragments were incubated at 37°C in a 5% CO₂ incubator in the enzyme solution for 18 hours. After centrifugation, the cells were resuspended in fresh media and were plated in 100mm culture dishes in DMEM containing 10% heat-inactivated fetal bovine serum, 2uM forskolin, and 10nM neuregulin. In Schwann cells cultured without forskolin, the nerve fragments were incubated with DMEM containing 10% serum for 3 days. Then the nerve fragments were prepared as previously described in media containing 10% serum with no forskolin or neuregulin β 1. The cells were harvested 24 hours after plating.

Patient information

Adult normal nerves were used as controls obtained from Dr. Wood. The diagnosis for NF1 was made by a neurosurgeon or a dermatologist based clinical symptoms, such axillary frecklings, café au lait macules. NF1 tumors were obtained from patients diagnosed with NF1. After diagnosis, the tumors were recovered surgically by Dr. John Shea (Loyola

University Medical Center, Department of Neurosurgery, Maywood, IL). Neurofibroma tumors were obtained from: NF-1 tumor of the left medial nerve of a 32 year old male (tumor 1); facial cutaneous neurofibroma from a 57 year old male (tumor 2); a plexiform neurofibroma from a 46 year old female (tumor 3); plexiform neurofibroma from a 60 year old female (tumor 4).

Proliferation assay

To measure cell proliferation, cells were replated in 96-well clusters at 5,000 cells per well. Cells were allowed to adhere to the substratum and then switched to serum free DMEM, containing the growth factors at the doses indicated in the text. Cell number was evaluated by using the colorimetric MTT assay (Boehringer-Mannheim, Indianapolis, IN) following the manufacturer's protocol. Absorbance was measured at 595nm and was shown to correlate with viable cell number under the conditions in this study.

Western Blotting

Cells cultured in DMEM supplemented with 10% FCS were lysed in RIPA (1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in phosphate buffer saline) containing a cocktail of protease inhibitors, PMSF, leupeptin and aprotinin. Tumor lysates were obtained by homogenizing the tissue in RIPA. Protein lysates were separated by electrophoresis in a 4 to 10% gradient polyacrylamide gel and transferred onto a PVDF membrane (Dupont-NEN, Boston, MA) for 1 hour at 80V. After blocking with a 5% nonfat dry milk solution for 30 minutes, the PVDF membrane was incubated in a solution containing the primary antibody overnight at 4°C. Following 4, 20-minute washes in PBS containing 1% Tween 20, the membrane was incubated with a horseradish peroxidase-conjugated, secondary antibody (Transduction Systems, San Diego, CA) for 1 hour at room temperature. After 3 washes with

PBS-T for 10 minutes each, immunoreactivity was evaluated using enhanced chemiluminescence (Dupont-NEN, Boston, MA).

Digital Fluorescence Ratio Imaging Of Intracellular Calcium Levels

NF1 Schwann cells and normal human Schwann cells were seeded onto 25mm coverslips in low-glucose DMEM with 10%FBS. Following serum starvation overnight, cells were loaded with 0.5 to 1uM of with the Ca^{2+} -sensitive indicator Fura2/AM (Molecular Probes, Eugene ,OR) in DMEM or HBSS for 20 minutes at 37°C. After washing the cells twice with PBS or HBSS, the coverslip was fitted in a holder with a maximum holding volume of 1ml. The holder was filled with 1 ml of warm HBSS or PBS and mounted onto the microscope stage of a Zeiss Axiovert 135 inverted microscope equipped with digital fluorescence microscopy. Fields of at least 20 cells were selected for Ca^{2+} measurements using a fluor x40 oil objective. Computer images of all of the cells were captured and digitally monitored using an ICCD camera. Recordings measured changes in fluorescence intensity in Schwann cells in a HEPES-buffered, balanced, salt solution (pH 7.2) or in PBS at room temperature. The excitation light from a xenon lamp alternated between 340nm (Ca^{2+} bound) and 380nm (Ca^{2+} -free) band-excitation filters, and the emission was measured at 520nm. Following 60 to 90 seconds of baseline measurements, PDGF BB or thapsigargin were added at the indicated time into the 1ml holding volume and assayed for a change in Fura-2 ratio. Fluorescence signals of at least 20 cells were acquired, stored, and analyzed using the Zeiss Attofluor RatioVision system. Increases in intracellular calcium were determined using the ratio method.

Statistics

Statistical analysis was performed using Sigma Plot [®]v2.0 software (SPSS[™]).

RESULTS

PDGF receptor β expression in NF1 tumors

The expression levels of PDGF R β in NF1 tumors from patients and normal peripheral nerves were compared. To determine PDGF growth-factor receptor levels in NF1 tumors, lysates from 4 different NF1 tumors were analyzed by immunoblotting using a PDGF R β antibody. All tumor samples have PDGF R β but 3 out of 4 samples showed high levels of PDGF R β when compared to normal adult peripheral nerve as illustrated in figure 1A. These results confirm that cells present within the NF1 tumors express high levels of the growth factor receptor. Although most cells present in the tumor are Schwann cells, other cells within the tumor may also have PDGF R β on their surfaces. However, we have previously reported (Badache *et al.*, 1998b) that Schwann cell lines derived from NF1 tumors overexpress PDGF receptors.

PDGF receptor β and neurofibromin expression in NF1 cell lines, non-NF1, and normal Schwann cells.

Badache *et al.* (1998b) reported that high levels of PDGF R β were present on 2 neurofibrosarcoma cell lines: ST88 and T265. The receptor levels of PDGF R β were studied in two additional NF1 cell lines: 90-8 and 88-3. To investigate levels of PDGF receptor on NF1 cell lines, 25 μ g of cell lysates were analyzed by immunoblotting using a PDGF R β antibody. The immunoblot revealed that all NF1 cell lines (T265, ST88, 90-8, and 88-3) have high levels of PDGF receptors on their surface (Figure 1B). However, normal human adult Schwann cells isolated from human peripheral nerves had PDGF R β expression levels comparable to that of the other 4 NF1 cell lines as shown in Figure 1B. These high receptor levels in normal adult Schwann cells may be attributed to the chronic elevation of intracellular

cAMP due to forskolin as suggested by Weinmaster et al., (1990). Interestingly, similar levels of PDGF R β levels on normal human adult Schwann cells were observed in the presence or in the absence of the cAMP elevating agent forskolin (Figure 1B). These high levels of growth factor receptor suggest that cAMP may not have any effects of growth expression levels of the PDGF receptor. In addition, the high levels of PDGF receptor β in NF1 cells are associated with the absence of neurofibromin while normal human adult Schwann cells have normal levels of neurofibromin (Figure 1C).

PDGF BB and downstream effectors in T265 cells

The differences between normal adult Schwann cells and NF1 Schwann cells may be related to pathways activated by PDGF BB, since levels of PDGF receptor expression are similar in both cell types. To investigate ERK or Akt phosphorylation induced by PDGF BB, NF1 cells were cultured in serum-free medium with 20 ng/ml PDGF BB for up to 120 minutes. Cell lysates were analyzed by immunoblotting using a phospho-specific ERK or Akt antibody. PDGF BB induced phosphorylation of ERK1 and ERK2 within 10 minutes of stimulation (Figure 2A). After 120 minutes, ERK1 and ERK2 phosphorylation decreased to basal levels. We used a non-phosphorylated ERK1 antibody to determine that protein levels were similar for each time point, as shown in Figure 2B.

In addition, PDGF BB induced phosphorylation of Akt within 10 minutes of stimulation as shown in Figure 2D. After 60 minutes, Akt phosphorylation decreased to basal levels. We confirmed equal protein loading for each time point with an antibody against the non-phosphorylated form of Akt (Figure 2E).

PDGF BB and downstream effectors in normal human adult Schwann cells

We postulated that PDGF BB activates different downstream effectors in normal human adult Schwann cells from those in NF1 Schwann cells. To investigate the ERK and Akt

phosphorylation, normal human adult Schwann cells were cultured in serum-free medium with 20 ng/ml PDGF BB for up to 120 minutes. Normal Schwann cell lysates were analyzed by immunoblots using a phosphospecific ERK and Akt antibody. PDGF BB induced phosphorylation of ERK after 10 minutes and the activation decreased to basal levels after 60 minutes (Figure 2C). In addition, PDGF BB cells induced Akt phosphorylation after 10 minutes in normal adult Schwann cells. Akt phosphorylation remained above basal levels after 120 minutes, as illustrated in Figure 2F.

Proliferation of NF1 Schwann cells and normal adult Schwann cells in response to PDGF BB

Despite activation of similar signaling molecules to NF1 Schwann cells by PDGF BB stimulation, human adult Schwann cells did not proliferate to the extent of NF1 Schwann cells in response to PDGF BB (figure 3). After 72 hours, PDGF BB induces almost a 2-fold increase in NF1 Schwann-cell proliferation (Badache *et al.*, 1998b), while human adult Schwann cells respond at a much slower rate (approximately a 120% increase). This difference in proliferation may be due to the activation of additional signaling pathways in NF1 Schwann cells compared to human Schwann cells.

PDGF BB increases intracellular calcium levels in NF1 Schwann cells

To determine real time intracellular calcium levels in NF1 Schwann cells, cells were incubated with 1uM of Fura 2/AM. Changes in relative fluorescence of the 380/340 ratio were monitored for increases in calcium levels in response to PDGF BB. The individual results of more than 20 cells are illustrated in Figure 5A. The average fluorescence of NF1 Schwann cells illustrated a rise in intracellular calcium levels in response to PDGF BB (Figure 4B). The influx of calcium reached a maximum 100 seconds after adding PDGF BB (Figure 4B). The

levels of calcium in normal human adult Schwann cells remain unchanged in response to PDGF BB but did respond to the addition of a 1 μ M of ionomycin, a Ca^{2+} elevating agent (Figure 4C). In media depleted of calcium cells still responded similarly to those in calcium containing media (data not shown) implying that the PDGF BB-induced rise in calcium levels was a result of intracellular calcium. Thapsigargin, an ATPase calcium-pump inhibitor, was used to block the reuptake of intracellular calcium and identify the source of the calcium increases. The addition of 1 μ M of thapsigargin to the T265 resulted in an increase Ca^{2+} levels and inhibited further releases of intracellular calcium with subsequent addition of PDGF BB (Figure 5A). Cells treated with vehicle control did not affect the release of calcium from intracellular storage in response to PDGF BB added subsequently (Figure 5B). Thus, these results are consistent with the view that the source of the PDGF BB-evoked calcium in NF1 Schwann cells derives from the intracellular calcium storage.

PDGF BB increases phosphorylation of CAMKII

The increase in intracellular calcium levels in Schwann cells may lead to activation of calcium-dependent proteins, such as CAMKII, contributing to the aberrant proliferation of NF1 Schwann cells. To investigate phosphorylation of CAMKII, T265 cells were cultured with serum-free medium with 20 ng/ml PDGF BB for up to 6 hours. Schwann-cell lysates were analyzed by immunoblotting using a phosphospecific CAMKII antibody. PDGF BB induced phosphorylation of CAMKII after 5 minutes with maximum phosphorylation after 120 minutes (Figure 6A). These results suggest that the activation of the PDGF receptor with its ligand PDGF BB induces the phosphorylation of the CAMKII.

We investigated the role of CAMKII in the proliferation of NF1 Schwann cells in response to PDGF BB. NF1 cells were cultured in serum-free medium with 20 ng/ml of PDGF BB in combination with the CAMKII inhibitor KN62 for 72 hours. The results show that the CAMKII

inhibitor decreased cell proliferation induced by PDGF BB in a dose-dependent manner with a maximal inhibition at 4uM (Figure 6B). These results reveal that proliferation of NF1 Schwann cells induced by PDGF BB is mediated by CAMKII of the Ca^{2+} signaling pathway.

DISCUSSION

Our findings indicate that freshly isolated tumors from NF1 patients express PDGF R β . In addition, we show that two additional Schwann-cell lines derived from NF1 tumors have high levels of PDGF R β on their surfaces. Surprisingly, normal human adult Schwann cells express PDGF receptor and neurofibromin, which is absent in NF1 Schwann cells. In normal human adult Schwann cells, receptor activation results in the phosphorylation of ERK and Akt. In addition, the proliferation of Schwann cells induced by PDGF BB is mediated by Akt of the PI3K pathway. Interestingly, activation of the PDGF R β is associated with an intracellular increase in calcium levels and phosphorylation of CAMKII in NF1 Schwann cells. Inhibition of the CAMKII with KN62 resulted in decreased cell proliferation.

Normal human Schwann cells isolated from adult peripheral nerves express PDGF receptors in both the presence and the absence of forskolin. In a previous report, Badache et al., (1998b) compared levels of PDGF receptors in NF1 Schwann cells with that of non-NF1 Schwann-cells (STS26T). However, in our report that PDGF R β levels on NF1 Schwann cells have compared to those of normal human adult Schwann cells explaining the discrepancy between the 2 studies.

In addition, the elevated levels of PDGF R β on normal human adult Schwann cells could be caused by the presence the elevating cAMP agent forskolin. Previously, Weinmaster et

al. (1990) reported that chronic elevations of cAMP with forskolin on rat Schwann cells induce high levels of PDGF R β . Similar to the case of rat Schwann cells, forskolin may also have induced high PDGF R β levels in normal adult Schwann cells. However, when forskolin was omitted from the isolation procedure, receptor levels in human adult Schwann cells were still high (see Figure 1B). This finding suggests that elevated PDGF R β levels are endogenously present on normal human adult Schwann cells.

It is interesting to note that levels of 2 growth factor receptors are affected by the loss of neurofibromin. Our laboratory reported that NF1 Schwann cells aberrantly express c-kit (Badache *et al.*, 1998a) and DeClue *et al.* (2001) reported that NF1 Schwann cells express EGF receptor on their surfaces. PDGF R β along with these 2 growth factor receptors may contribute to the aberrant proliferation of NF1 cells. Interestingly, the Erb-B receptors, which contribute to proliferation of normal Schwann cells are absent in the transformed state (Badache *et al.*, 1998b; DeClue *et al.*, 2000).

Our findings suggest that PDGF BB stimulates the phosphorylation of Akt of the PI3K pathway in NF1 Schwann cells and in normal human adult Schwann cells. A recent study reported that the activation of Akt is important for Schwann cell proliferation and survival (Maurel *et al.*, 2000). In addition, IGF1 prevents Schwann-cell apoptosis through the PI3K (Delaney *et al.*, 1999). Overall, these studies confirm that Akt protein has been shown to mediate important physiological functions in normal Schwann cells. Similarly, our results provide evidence that activation of Akt is equally critical for both normal human adult Schwann cell proliferation and NF1 Schwann-cell proliferation contributing to the development of NF1 Schwann cell tumors.

This research also examined the extent to which the ERK pathway was aberrantly regulated in NF1 Schwann cells. We showed that PDGF BB induces ERK phosphorylation in both NF1 Schwann cells and in normal human adult Schwann cells. No differences between the two cell types were observed in the ERK pathway. These results suggest that PDGF BB activates the PI3K and ERK pathway in a similar manner in normal human adult Schwann cells and in NF1 Schwann cells in response to PDGF BB. Thus, the loss of neurofibromin in NF1 Schwann cells has not affected the signaling of either of these two pathways. It is consistent with the view that the cause for the increased proliferation of NF1 Schwann cells in response to PDGF BB may be due to abnormal signaling of other metabolic pathways, such as the cAMP or the calcium pathway.

The elevation of intracellular calcium levels in response to PDGF BB is the major transduction signaling difference between normal and NF1 Schwann cells. The release of calcium from internal storage was observed in two cell lines (T265 and ST88). Calcium may mediate the proliferation of NF1 Schwann cells induced by PDGF BB. In addition, the downstream effector of calmodulin, CAMKII, activated by the release of calcium was phosphorylated in response to PDGF BB. Taken together, these results suggest that calcium plays a role in NF1 Schwann cell proliferation.

Studies have shown that calcium has a variety of physiological effects including proliferation and apoptosis (Berridge *et al.*, 1998). For instance, in the central nervous system calcium induces apoptosis in neurons and oscillations of calcium levels in neuronal cells are involved with long term potentiation. In the immune system, calcium mediates the proliferation of T and B cells. In glial cells, the function of calcium in normal Schwann cells has already been documented in several reports, but this is the first study characterizing calcium in NF1 Schwann cells.

PDGF BB did not elevate calcium in normal Schwann cells. Presently, only two compounds, ATP and serotonin, increase intracellular calcium in Schwann cells. ATP was the first compound to increase calcium levels in normal Schwann cells (Lyons *et al.*, 1995; Ansselin *et al.*, 1994). Later, the neurotransmitter serotonin increases calcium levels through HT2a receptors (Yoder *et al.*, 1996). The physiological role of calcium in response to ATP has been elucidated by Stevens *et al.*, (2000) using the Schwann cell and DRG co-culture. With the *In vitro* model, they were able to demonstrate that ATP is released from neurons following electrical stimulation. Upon binding to the purinergic receptor on Schwann cells, ATP induces elevations of calcium levels resulting in decreased Schwann cell proliferation.

Our results indicate that calcium is involved in proliferation of NF1 Schwann cells. It may be that the loss of neurofibromin expression switches the cellular function of calcium favoring conditions for proliferation in NF1 Schwann cells over the conditions inhibiting proliferation in normal Schwann cells. These findings suggest that neurofibromin regulates signaling pathways and its loss results in aberrant intracellular signaling in Schwann cells leading to the development of NF1 tumors. The increases in intracellular calcium levels in conjunction with the activation of the PI3K or the ERK signaling pathway may result in the proliferation of NF1 Schwann cells. Therefore, the increased proliferation of NF1 Schwann cells is due to aberrant intracellular signaling in NF1 Schwann cells and not due to increased levels of PDGF R β levels.

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FIGURE LEGENDS

Figure 1. Western blot analysis of PDGF Receptor β levels in NF1 tumors, and in NF1 Schwann cells and normal adult Schwann cells. Four fresh NF1 tumor samples and one human peripheral nerve samples were lysed with RIPA and 25 ug of protein were analyzed by immunoblotting with a PDGF R β antibody (A). This immunoblot is a representative sample of the experiment, which was repeated twice with similar results. Cells were lysed and 50ug of proteins were analyzed by immunoblotting with PDGF R β (B) and neurofibromin (C) antibodies. These immunoblots are a representative a respresentative sample of the experiment, which was repeated three times with similar results.

Figure 2. PDGF BB induces phosphorylation of ERK and Akt in NF1 Schwann cells. The NF1 Schwann cells or normal human adult Schwann cells were cultured with 20 ng/ml of PDGF BB for up to 120 minutes. NF1 cells were lysed and 25 ug of proteins were analyzed by immunoblotting with an antibody specific for the phosphorylated form of ERK (A) or Akt (D) and the non-phosphorylated form of Erk (B) or Akt (E). Normal human adult Schwann cell lysates were analyzed by immunoblotting with an antibody specific for the phosphorylated form of ERK (C) or Akt (F). These immunoblots are a representative sample of the experiment, which was repeated three times with similar results.

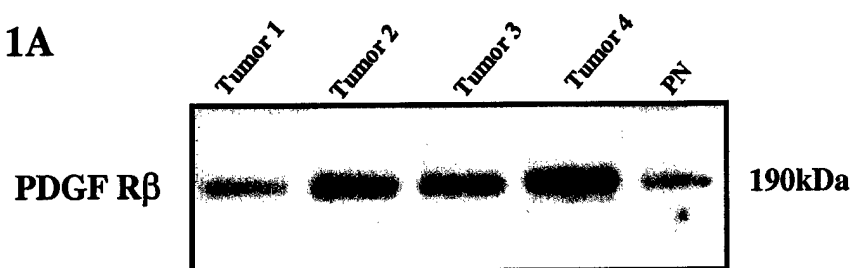
Figure 3. PDGF BB induces higher levels of proliferation in NF1 Schwann cells than in normal human adult Schwann cell. Normal human Schwann cells were cultured in serum-free medium in the presence of increasing concentrations of PDGF BB (5 to 40 ng/ml) for 72 hours. Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. The values are a representative sample of the experiment, which was repeated three times with similar results. Values are expressed as average fold increase over controls. Error bars represent the standard deviation of three samples.

Figure 4. PDGF BB elevates intracellular calcium levels in NF1 Schwann cells, but not in normal adult Schwann cells. Schwann cells were loaded with 1 μ M Fura-2/AM for 20 minutes and levels of intracellular calcium were measured using the 340/360 ratio. Individual cell responses are depicted in (A); the mean of the responses of twenty cells are depicted in (B). Normal adult Schwann cells were loaded with 1 μ M Fura-2/AM for 20 minutes and levels of intracellular calcium were measured using the 340/360 ratio. 20ng/ml of PDGF BB was added to cells and a calcium ionophore (1 μ M ionomycin) was added subsequently (C). This calcium ratio tracing is a representative sample of the experiment, which was repeated three times with similar results.

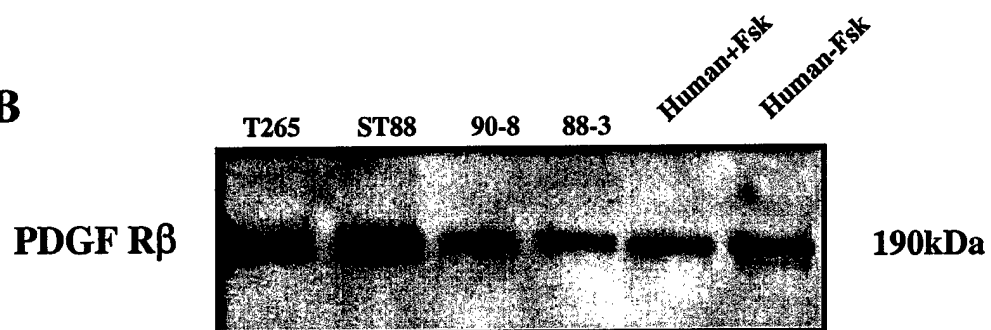
Figure 5. Increases in intracellular calcium levels are due to the release of calcium from the cell internal storage. Schwann cells were loaded with 1 μ M of Fura-2/AM for 20 minutes and levels of intracellular calcium were measured using the 340/360 ratio. Calcium responses with 1 μ M thapsigargin and 20 ng/ml PDGF BB added subsequently are depicted in (A). Calcium responses with vehicle control and 20 ng/ml PDGF BB added subsequently are illustrated in (B). These calcium ratio tracings are a representative sample of the experiment, which was repeated three times with similar results.

Figure 6. CAMKII is activated by PDGF BB and mediates the proliferation in NF1 Schwann cells induced by PDGF BB. The NF1-derived Schwann cells were incubated with 20 ng/ml of PDGF BB for up to 90 minutes. Cells were lysed and 25 μ g of proteins were analyzed with an antibody specific for the phosphorylated form of CAMKII (A). This immunoblot is a representative sample of the experiment, which was repeated three times with similar results. T265 cells were cultured in serum-free medium in the presence of 20 ng/ml of PDGF BB with increasing concentrations of the CAMKII inhibitor, KN62, for 72 hours (B). Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. Values are expressed as mean of five samples from a representative sample of the experiment, which was repeated three times with similar results. Error bars represent \pm standard deviation. The data were determined to be statistically significant using ANOVA ($F(4,20)=46.238$). Post-hoc comparisons were determined using Tukey's LSD (*, significantly different from control, $P<0.05$).

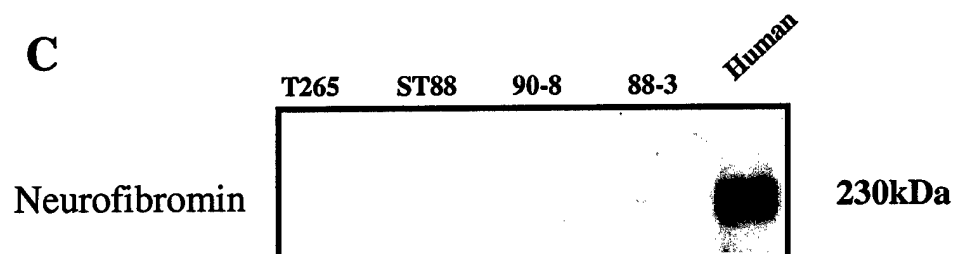
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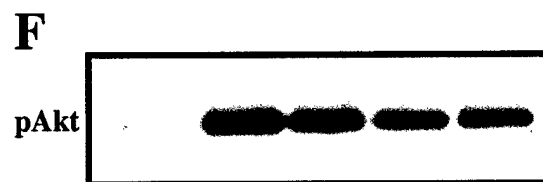
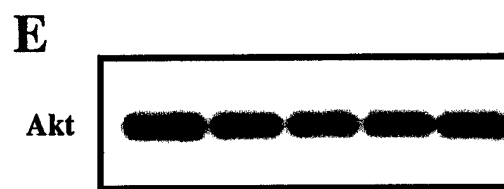
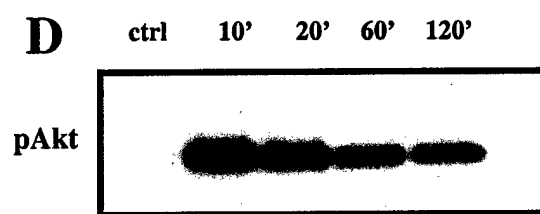
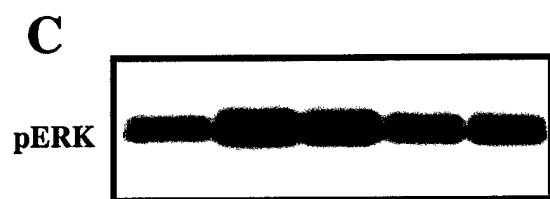
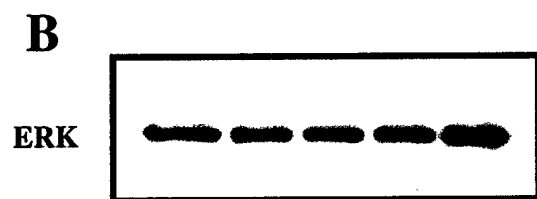
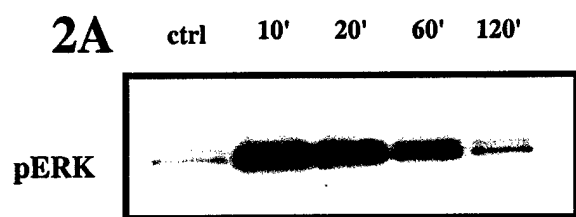


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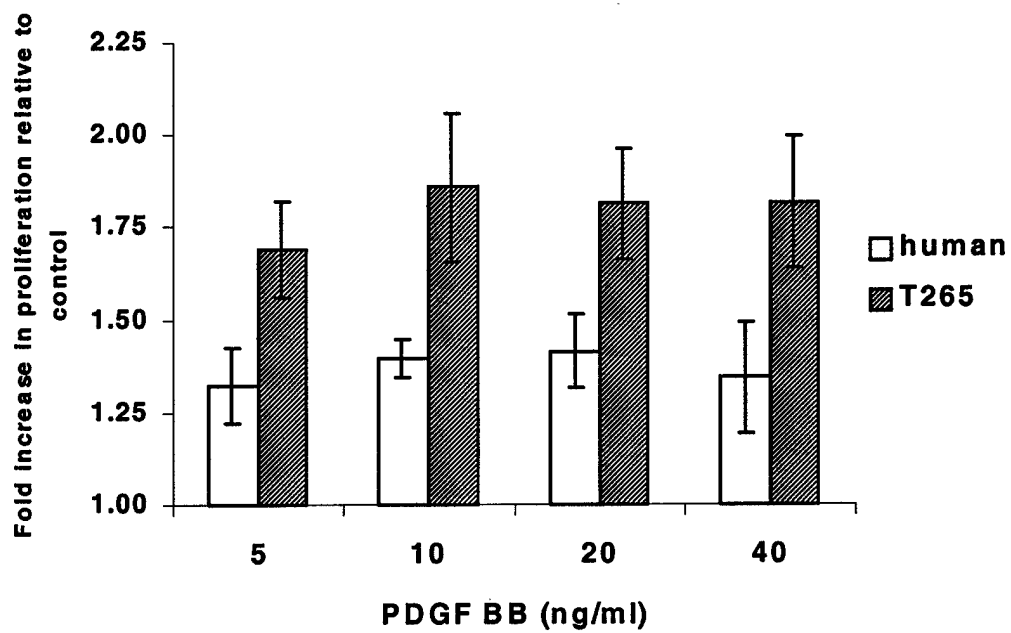


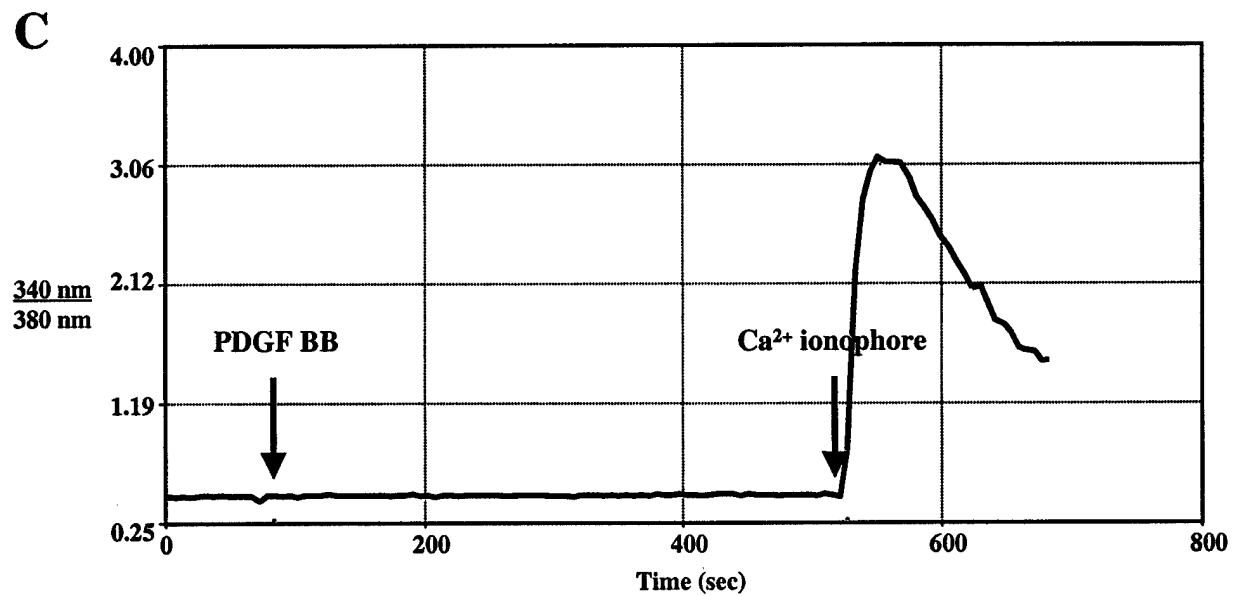
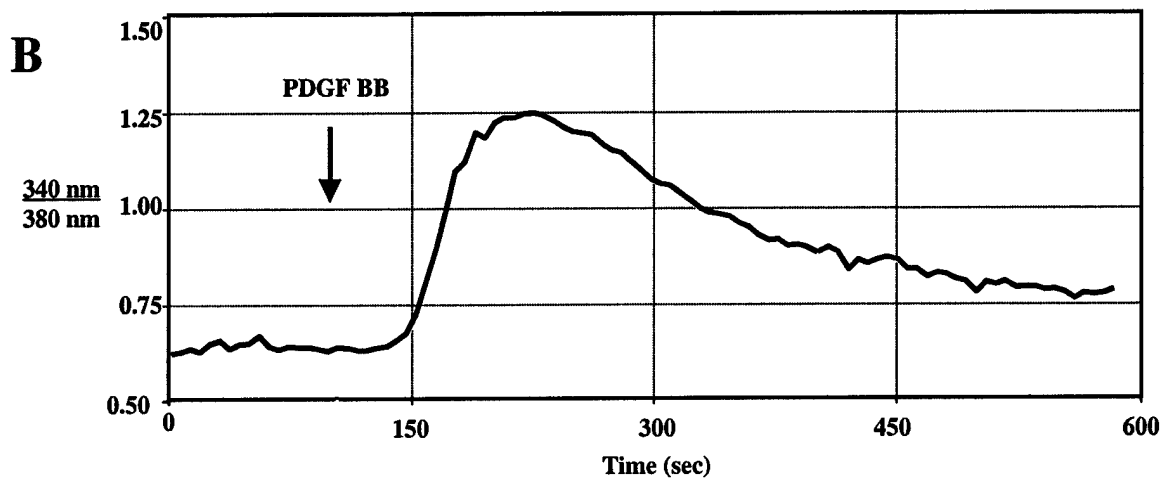
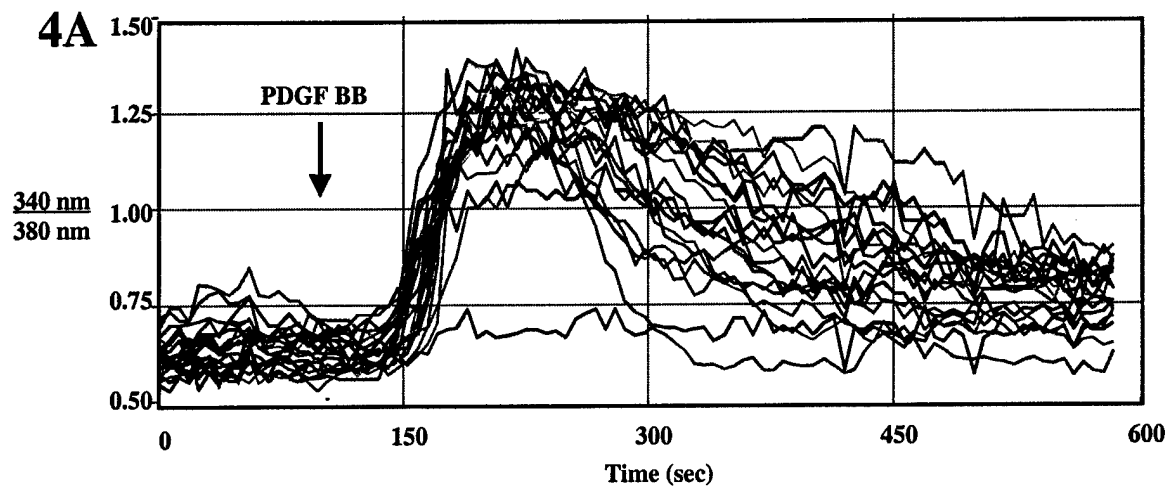
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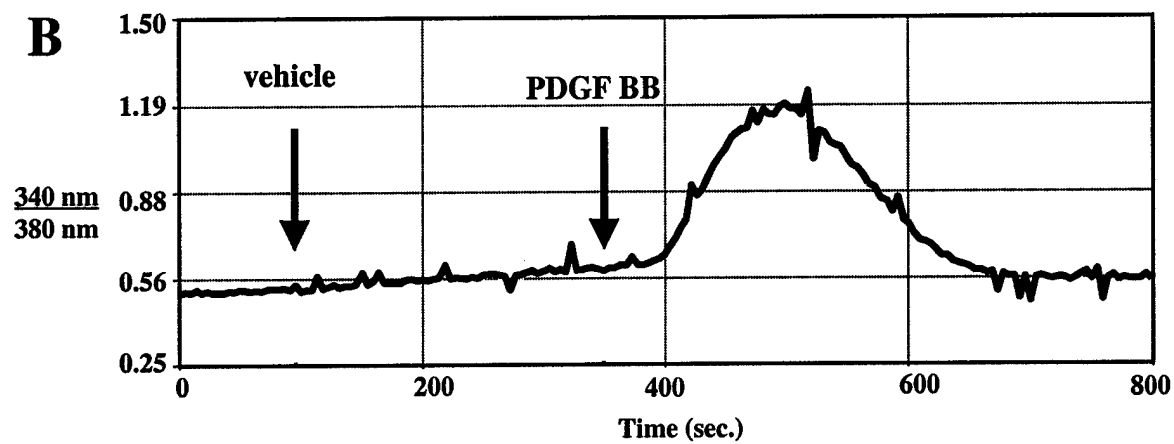
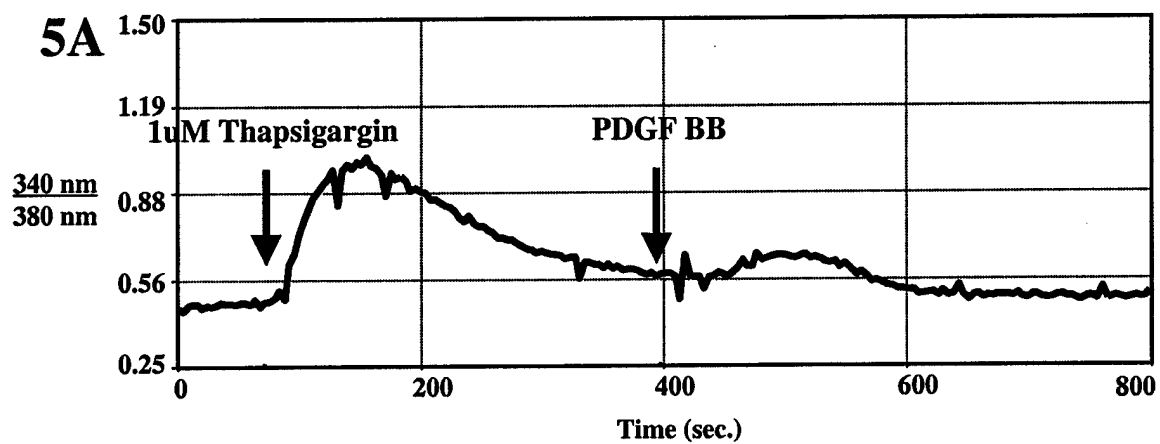




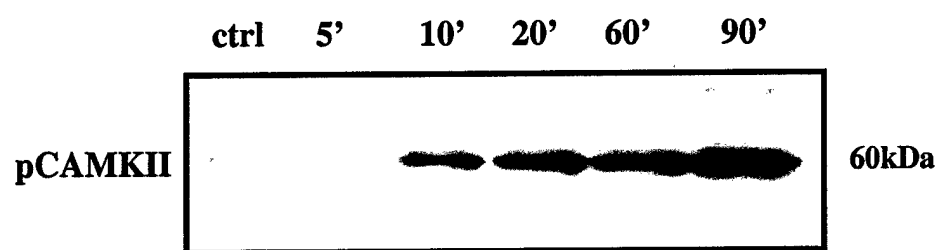
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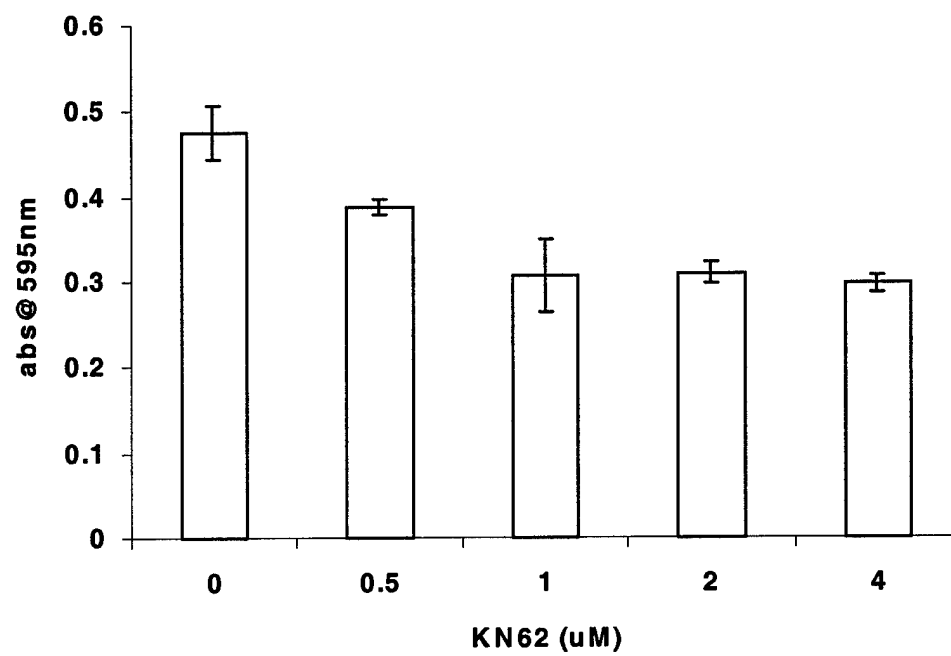




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C-KIT RECEPTOR EXPRESSION IN NORMAL HUMAN SCHWANN CELLS AND SCHWANN CELL LINES DERIVED FROM NF1 TUMORS

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Running title: c-Kit in Normal and Transformed Human Schwann Cells

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Abstract

Neurofibromatosis type 1 (NF1) is a genetic disorder characterized by abnormal proliferation of Schwann cells in relatively benign (neurofibromas) or malignant (neurofibrosarcoma) tumors. Our laboratory has reported that neurofibrosarcoma-derived Schwann cells contain high levels of the tyrosine kinase receptor c-kit. Although stimulation of c-kit in these cells only slightly increased the basal Schwann cell proliferation (Badache et al, 1998a), we now investigate the relationship of c-kit and neurofibromin expression and the potential role of c-kit in Schwann cell development. Neurofibromin and c-kit are inversely regulated in rat sciatic nerves during development: c-kit is virtually absent in adult nerve whereas neurofibromin is strongly expressed in adult nerve. Normal adult human Schwann cells contain neurofibromin and do not express c-kit, while human Schwann cell lines derived from NF1 tumors express c-kit but do not express neurofibromin. In cultured neonatal rat Schwann cells, activation of c-kit by stem cell factor prevents programmed cell death via the activation of Akt but does not induce Schwann cell proliferation or differentiation. These results indicate an anti-apoptotic role for c-kit during Schwann cell development. Abnormal expression of this receptor in NF1-derived Schwann cells may contribute to tumor growth via inhibition of apoptosis.

Keywords: neurofibromatosis type I, Schwann cells, c-kit, Stem cell factor

Introduction

Many growth factors interact with specific cell surface receptors belonging to the receptor tyrosine kinase (RTK) superfamily. Members of the RTK superfamily possess both shared and unique structural subdomains (Yarden and Ullrich, 1988). The proto-oncogene, c-kit, belongs to the RTK superfamily and is a member of the same receptor subfamily as the platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1) receptors (Yarden et al, 1987 and Qui et al, 1998). This RTK subfamily is characterized by the presence of five immunoglobulin-like motifs in the extracellular domain and an insert that splits the cytoplasmic kinase domain into an adenosine triphosphate (ATP)-binding region and a phosphotransferase domain (Yarden et al, 1988). C-kit and its ligand, stem cell factor, constitute an important signal transduction system regulating cell growth and differentiation in hematopoiesis, gametogenesis, melanogenesis (for review see Broudy 1997). Since we have previously reported the presence of high levels of c-kit in NF1 derived Schwann cells (Badache et al, 1998), it is possible that c-kit and stem cell factor play a role in the pathogenesis of neurofibromatosis type 1.

Neurofibromatosis type 1 (NF1) is a genetic disorder affecting approximately 1 in 3000 individuals that manifests with various phenotypic features including neurofibromas, café-au-lait spots, axillary freckling, Lish nodules, optical gliomas, and learning disabilities (Friedman, 1999). Neurofibromas are benign peripheral nerve sheath tumors that consist of a majority of Schwann cells, but also contains fibroblasts, perineurial cells, and mast cells embedded in an abundant extracellular matrix. Most importantly, Schwann cells are the major cell type responsible for the formation of malignant neurofibromas in NF patients (Friedman, 1999).

In hematopoietic cells, abnormal proliferation of both immature and lineage restricted progenitor populations derived from Nf-/- mice occurs in response to SCF (Zhang et al., 1998).

In addition, mast cells derived from Nf1^{+/+} mice display increased cell proliferation, survival, colony formation in response to SCF (Ingram et al., 2000). Interestingly, mast cells are present in neurofibromas and have been implicated in promoting Schwann cell growth through the release of various factors (Ricardi et al., 1992). These studies may explain hematopoietic disorders associated with the loss of neurofibromin. Children with NF1 are predisposed to juvenile chronic myelogenous leukemia (JCML) and other malignant myeloid disorders, and heterozygous NF1 knockout mice spontaneously develop a myeloid disorder resembling JCML (Shannon et al., 1994; Jacks et al., 1994). Therefore, c-kit receptor may be important during development for numerous cell types, such as progenitor cells, mast cells, and possibly Schwann cells. On the other hand, neurofibromin is a tumor suppressor that regulates signal transduction pathways at a later stage of development and in the adult. Therefore, loss of neurofibromin in the adult results in the development of Schwann cell tumors and disorders of the hemapoetic system, such as leukemia.

We have observed that Schwann cells derived from neurofibrosarcomas abnormally express high levels of the receptor tyrosine kinase c-kit, which may contribute to Schwann cell proliferation (Badache et al, 1998). We postulated that c-kit is inappropriately re-expressed by adult Schwann cells present in neurofibrosarcomas; however, during normal development of Schwann cells the receptor may play an important role. We now report that c-kit is developmentally regulated in rat sciatic nerves and that activation of this receptor prevents Schwann cell apoptosis during development. The SCF induced activation of c-kit expressed in cultured Schwann cells causes phosphorylation of the downstream effector Akt known to be activated in an anti-apoptotic pathway.

Materials and Methods

Cell lines and Reagents

Recombinant stem cell factor (SCF) and $\beta 1$ neuregulin were a gift from Amgen (Thousand Oaks, CA). Forskolin was from Calbiochem (La Jolla, CA). The cell lines ST88-14, T265, STS 26T used in this study have been previously described (Badache et al., 1998). The 90-8 and 88-3 cell lines were obtained from Dr. Jeff DeClue and have been previously described (DeClue et al. 1992).

Schwann cell Isolation

Primary neonatal rat Schwann cell cultures were established essentially as described by Brockes and colleagues (1979). Sciatic nerves were removed from 3-day rat pups, digested for 2 hours in 0.03% collagenase (Serva) at 37°C, and triturated thoroughly to achieve dissociation. Cells were cultured as monolayers in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and 100ug/ml streptomycin, and maintained at 37°C in a humid atmosphere of 10% CO₂/90%air. Contaminating fibroblasts were inhibited by 72 hours treatment with 10uM cytosine arabinoside.

Primary human adult Schwann cell cultures were established as described by Casella et al., (2000) (Miami Project, University of Miami, Miami, Fla).

Sciatic nerves

Rat sciatic nerves were extracted from neonatal and adult animals. The nerves were lysed in RIPA buffer (1% triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, SDS, in phosphate buffered saline, PBS, pH 7.2) containing 1mM sodium orthovanadate and a cocktail

of protease inhibitors. Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA).

Proliferation assay

To measure cell proliferation, cells were replated in 96-well clusters at 5,000 cells per well. Cells were allowed to adhere to the substratum and then switched to serum free DMEM, containing the growth factors at the doses indicated in the text. Cell number was evaluated by using the colorimetric MTT assay. MTT labeling reagent (0.5mg/ml, Sigma, St Louis, MO) were added to Schwann cell cultures for 4 hours (humidified atmosphere, 5% CO₂, 37°C). Following overnight incubation with a solubilization solution (10% SDS in 0.01M HCL), absorbance was measured at 595nm using an automated microplate reader EL311sx (Bio-Tek Instruments, Inc., Winooski, VT). The increase in absorbance was shown to correlate with viable cell number under the conditions of this study.

Western blotting

Cells cultured in DMEM supplemented with 10% FCS or rat sciatic nerves were lysed in RIPA buffer (1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in phosphate buffer saline) containing a cocktail of protease inhibitors, PMSF, leupeptin, aprotinin (Calbiochem). Protein lysates were separated by electrophoresis in a 4 to 10% gradient polyacrylamide gel (InVitrogen) and transferred onto a PVDF membrane (Dupont, NEN, Boston, MA) for 1 hour at 80V. After blocking with a 5% nonfat dry milk solution for 30 minutes, the PVDF membranes were incubated overnight in the presence of the primary antibody (anti-kit from Oncogene product, anti-neurofibromin from Santa Cruz Biotechnology, anti-phospho Akt and anti-Akt

from Cell Signaling Systems), washed 4 times in PBS containing 1% Tween 20 for 20 minutes, and were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour (Transduction Systems, San Diego, CA). After 3 washes with PBS-T for 10 minutes, the immunoreactivity was detected by enhanced chemiluminescence (Dupont-NEN, Boston, MA).

RT/PCR

RT/PCR reactions were performed as previously described by Clive et al., (1998). C-kit oligonucleotides were synthesized for our laboratory by Gibco BRL. Sequences of PCR primers are as following: c-kit upstream: 5'-TGA AGC CGG GGA CAC CAT-3' downstream: 5'-GTT TGC GGC ACA GAC ACC-3'; P0 primers upstream: 5'-GCT CTT CTC TTC TTT GGT GCT GTC C 3'; downstream: 5'-GGC GTC TGC CGC CCG CGC TTC G3'.

TUNEL assays

25,000 cells were plated on 8-chambered glass slides (Nunc). The cells were cultured in serum free media or with SCF or $\beta 1$ neuregulin (50ng/ml) in serum media. After rinsing with PBS they were fixed with a 1% formaldehyde solution for 20 minutes. Following 2 washes with PBS, the cells were rinsed with tap water. The TUNEL reactions were carried out at 37°C for 60 minutes. TUNEL mixes consisted of 1xTdT buffer (GibCo), 0.75U/ul of TdT (GibCo), 100uM B-14-cCTP (GibCo). Following 2 washes with PBS, the cells were incubated for 30 minutes with 25ug/ml of FITC conjugated to avidin (GibCo) in staining buffer (4x SSC, 0.1%NP40, and 5% non-fat dry milk). The slides were mounted with Vectashield (Vector) and visualized with a fluorescent microscope at 520 nm. The percentage TUNEL positive cells were determined by counting approximately 800 cells for each condition.

STATISTICS

Statistics were determined using Sigma Plot 2.0 software.

Results

Developmental Regulation of c-kit in Rat Sciatic Nerves

To investigate c-kit expression in intact sciatic nerves as a function of development, rat sciatic nerves were obtained from several postnatal development ages, and their solubilized protein was analyzed by western blotting. C-kit receptor levels were the highest in the first 5 postnatal days and declined to the lowest level in the adult (**Figure 1, Panel A**). As expected, expression of P0 protein, a marker for Schwann cell differentiation, was lacking during early postnatal days and reached high levels at postnatal day 16 (**Figure 1, Panel B**). Interestingly, neurofibromin expression was also developmentally regulated as it was detected in the adult but not in younger sciatic nerves as shown in **Figure 1, Panel C**. These results strongly support the idea that there is an inverse relationship between the expression of neurofibromin and c-kit. Together, these results suggest that in the intact sciatic nerve the expression of c-kit is inversely correlated with that of P0 protein.

Expression of c-kit and neurofibromin in neurofibrosarcoma cell lines and in normal adult human Schwann cells

We have previously reported that c-kit was aberrantly expressed in 2 neurofibrosarcoma cell lines: ST88 and T265 (Badache et al. 1998). We now extend this observation by analyzing two additional NF1 Schwann cell lines 90-8 and 88-3 for c-kit. Lysates of these cells were analyzed by immunoblotting using antibodies against c-kit and neurofibromin. As shown in **Figure 2A**, cell lines derived from neurofibrosarcomas displayed high expression levels of c-kit

compared to that of the cell lines derived from non-NF1 tumors, the STS26T. In addition, normal human adult Schwann cells did not contain c-kit receptor in contrast to the high levels of c-kit receptor in the neurofibrosarcoma-derived T265 cell line (**Figure 2, Panel A**).

Interestingly, c-kit protein expression in neonatal rat Schwann cells was much lower than cell lines derived from neurofibrosarcomas, such as T265 and ST88 cells.

The level of expression of c-kit is inversely correlated to that of neurofibromin in NF1, non-NF1, and normal human adult Schwann cells (**Figure 2, Panel B**). These results indicate that cell lines derived from neurofibrosarcomas do not express neurofibromin as expected from the etiology of NF1, but they do express c-kit. Normal human adult Schwann cells express normal level of neurofibromin but do not express any c-kit (**Figure 2, Panel B**).

Akt and ERK1/2 phosphorylation after SCF stimulation of neonatal Schwann cells

The PI3K and ERK pathways are two transduction pathways that can be activated by tyrosine kinase receptors. We investigated the phosphorylation of Akt via PI3K pathway and the phosphorylation ERK1/2 via the ERK pathway when kit is activated with its ligand stem cell factor (SCF) in cultured neonatal Schwann cells. To measure Akt phosphorylation, Schwann cells were cultured in the presence of 50ng/ml of SCF for 10 to 120 minutes. Akt phosphorylation levels were analyzed by immunoblotting using an anti-phosphorylated Akt antibody. SCF induced phosphorylation of Akt within 10 minutes; the phosphorylated Akt reached a maximum after 20 minutes, as illustrated in **Figure 3, Panel A**. After 60 minutes of stimulation, Akt phosphorylation decreased but remained above the level of phosphorylated Akt found in of control non stimulated SC. This finding suggests that Akt is a downstream effector of kit in normal Schwann cells. Interestingly, immunoblotting studies revealed that SCF induced a

weak transient phosphorylation of ERK1/2, which was at a maximum level at 10 minutes and decreased to basal levels after 120 minutes (**Figure 3, Panel B**). Levels of non-phosphorylated Akt and ERK1/2 are similar among all time points (data not shown).

Effects of SCF on Schwann cell proliferation

After finding the signaling molecules activated by the c-kit receptor, we wanted to determine the functional role of c-kit in rat neonatal Schwann cells. Similar to what we have previously found in NF1 derived SC, we first looked at the ability of SCF to induce the cell proliferation of cultured neonatal Schwann cells (Badache et al., 1998a). To investigate the proliferative effect of SCF on primary rat Schwann cells, cells were cultured in the presence of 0, 25, 50, and 100ng/ml of SCF for 4 days. No increase in cell number was observed with cells incubated with SCF compared to control cells (**Figure 4, Panel A**). These results suggest that SCF by itself does not induce cell proliferation. To determine the possibility that SCF synergizes with other growth factors, such as β 1 neuregulin, to induce Schwann cell proliferation, cells were cultured with increasing concentrations of SCF in the presence of 20ng/ml of β 1 neuregulin for 4 days. SCF did not synergize with β 1 neuregulin, the most potent growth factor for Schwann cells (**Figure 4, Panel A**). These results indicate that SCF may not be a mitogen or a co-mitogen for Schwann cell proliferation.

Effects of SCF on Schwann cell differentiation

Since SCF does not have any effects on cell proliferation, we investigated its possible role in the differentiation of Schwann cells by examining the possibility of SCF to induce P0 mRNA using semi-quantitative PCR as described previously (Clive et al., 1999). To determine

expression of P0 mRNA cells were cultured in presence of 50ng/ml of SCF up to 72 hours; mRNA was extracted from Schwann cells and analyzed for the 603 base pair P0 products using RT/PCR. The levels of P0 mRNA were normalized with those of GAPDH, which were run concomitantly for each condition (data not shown). SCF did not increase P0 mRNA at any time points (**Figure 4, Panel B**). The expression of P0 mRNA in Schwann cells cultured with SCF remained unchanged compared to those of control Schwann cells. These results are consistent with the view that SCF does not affect Schwann cell differentiation.

Effects of SCF on Schwann cell apoptosis

To determine the effects of SCF on Schwann cell survival, we performed TUNEL assays on Schwann cells cultured for 72 hours in serum free media either in the presence of SCF or neuregulins. As expected, in serum free-media numerous Schwann cells were TUNEL positive (**Figure 5, Panel B**), and no staining was observed when the TdT enzyme was omitted from the TUNEL mixes (**Figure 5, Panel A**). However, the presence of SCF (50ng/ml) decreased the number TUNEL positive cells relative to controls (**Figure 5, Panel C**), indicating that SCF is somewhat effective in preventing Schwann cell apoptosis. As a positive control for Schwann cell survival, the cells were cultured in serum free media with $\beta 1$ neuregulin (50ng/ml). We detected more cells but no apoptotic cells were observed (**Figure 5, Panel D**). The number of apoptotic cells as a percentage of total cells are presented in **Figure 5, Panel E**. In serum free media, 50% of cells were TUNEL positive. At 50ng/ml of SCF, only 30% of Schwann cells were TUNEL positive. However, a lower concentration of SCF (25ng/ml) did not rescue Schwann cells from apoptosis. Interestingly, the addition of SCF in combination with the PI3K inhibitor LY294002 (10uM) increased the levels of apoptotic cells to those of controls

suggesting SCF signaling is mediated by the PI3K pathway. These results indicate that SCF promotes neonatal Schwann cell survival through the PI3K pathway.

Discussion

In this study, we have shown that c-kit is developmentally regulated in rat sciatic nerves. C-kit expression is at its highest level during postnatal development and at its lowest level in the adult. C-kit levels in whole nerve are inversely related to the level of P0 expression. In intact sciatic nerves neurofibromin expression is present in the adult but absent in neonates. In addition, cultured neonatal rat Schwann cells express c-kit protein. Upon the addition of SCF, c-kit activation results in the strong phosphorylation of Akt but weak phosphorylation of ERK. Functionally, the activation of c-kit by SCF prevents Schwann cell apoptosis but does not affect proliferation or differentiation.

Schwann cells express many growth factor receptors on their surface. The PDGF receptor has been well studied in Schwann cells, but it is not developmentally regulated. Its expression is as high in neonate as in the adult (Eccleston et al., 1993). The Erb-B receptor family is another type of Schwann cell surface growth factor receptor that is regulated during development. Similar to c-kit, Erb-B2 expression is high in neonatal sciatic nerves but is low in the adult (Cohen et al., 1992). In addition, Erb-B3 is developmentally regulated similar to Erb-B2 (Dang, unpublished observations). Furthermore, NGF is another growth factor receptor, which is regulated during development (Lemke et al., 1988). The developmental regulation of c-kit expression is similar to that of p75 expression. Both receptor expression levels are high in rat sciatic nerves until postnatal day 6 and decrease to low levels at postnatal day 14 and in the adult

(Tikoo et al., 2000). At the time when both receptor expression levels decrease, P0 expression increases to maximal level in the adult.

Interestingly, neurofibromin is absent in neonatal sciatic nerves, but is present in the adult. Its expression is high in the neonatal stages and disappears in the adult. These results suggest that the lack of neurofibromin expression correlates with the proliferation of Schwann cells. Both Erb-B2 and p75 receptor expressions are inversely correlated with that of P0 protein. In neonates c-kit expression is high during the proliferating stages of Schwann cells, but is low during the differentiating stages of Schwann cells in the adult. These data are consistent with the view that c-kit expression may be important during the survival phase of Schwann cells during development.

In this study, we report that c-kit is expressed by 2 additional NF1 cell lines, the 90-8 and 88-3, which are missing neurofibromin (Badache et al., 1998; DeClue et al., 1992). The expression of c-kit correlates with the absence of neurofibromin in Schwann cells derived from NF1 tumors. These observations are consistent with our previously reported studies (Ryan et al. 1994 and Badache et al. 1998). However, we report that neonatal rat Schwann cells express low c-kit receptor levels on their surface compared to that of NF1-derived Schwann cells which contain high levels of c-kit. These results are in contrast to the findings reported by Ryan and co-workers, who did not c-kit protein expression in neonatal rat Schwann cells (Ryan et al., 1994). The difference may be explained by the use of different antibodies in different techniques (Ryan et al., 1994). In addition, Badache and co-workers failed to detect c-kit in normal Schwann cells because the amount of protein analyzed was two fold less and the time for autoradiography was not sufficiently long enough for c-kit detection. These technical discrepancies may explain the differences observed between the reports. In conclusion,

increased amount of proteins loaded for immunoblotting and longer exposure during autoradiography explain that c-kit levels are low but detectable in neonatal rat Schwann cells.

Interestingly, neonatal sciatic nerves do not express neurofibromin, but cultured neonatal rat Schwann cells express high levels of neurofibromin. During Schwann cell isolation, Schwann cells lose axonal contact and cease to proliferate. Concomitantly, they increase the expression of neurofibromin, which correlates with the normal non-proliferative stage of Schwann cells as in the adult. A previous report has carefully shown that Schwann cells freshly isolated from neonatal rat sciatic nerves proliferate minimally unless cultured with forskolin and growth factors, such as neuregulins (Rahmatullah et al., 1998). Similar to our findings, Wrabetz et al. (1995) also reported that cultured neonatal rat Schwann cells express neurofibromin. In contrast to our studies, they investigated the regulation of neurofibromin mRNA expression and not neurofibromin protein expression in intact rat sciatic nerves. These discrepancies may explain the differences reported between the 2 studies.

Schwann cells undergo apoptosis when incubated in serum free media over 3 days (Syroid et al., 1998; Delaney et al., 1999). Schwann cell apoptosis is mediated by caspase activities, and the cells can be rescued by IGF (Delaney et al., 1999). Salzer et al. (2000) reported that neuregulins promote Schwann cell survival through the phosphorylation of Akt. Similarly, our results show that SCF is able to rescue Schwann cells from programmed cell death via the activation of Akt. However, SCF is not as potent as neuregulins that have already documented effects on Schwann cell survival (Syroid et al., 1996; Maurel et al., 2000). Our findings suggest that the difference between the two growth factors may be that neuregulins activate several signaling pathways, including MAPK and PI3K, while SCF favors the activation

of PI3K. Taken together, the activation of c-kit by SCF prevents Schwann cell death during development and contributes to the survival of NF1 Schwann cells.

Several studies have reported that neurofibromin regulates Ras due to its homologous sequence to GTPase activating protein, GAP, (DeClue et al., 1992). This sequence represents only a small fraction of the neurofibromin protein, while the majority of the Nf1 gene product has yet to be characterized. Besides Ras, it is possible that neurofibromin may play a role in the proliferation of Schwann cells during development. Wrabetz et al. (1995) reported the lack of regulation for NF1 mRNA expression but did not investigate neurofibromin protein in rat sciatic nerves. In this study we report that neurofibromin is developmentally regulated and expressed only in adult sciatic nerves. Together with Wrabetz and colleagues, our findings suggest that neurofibromin is translationally regulated in Schwann cells. Neurofibromin expression is absent in human fetal Schwann cells but present in adult human Schwann cells (Dang, unpublished observations). These observations are consistent with the idea that neurofibromin is related to the suppression of proliferation. It is absent during the proliferating stages of Schwann cells but is present when Schwann cells stop proliferating. The loss of neurofibromin in combination with other factors results in the transformation of adult Schwann cells leading to the development of neurofibromas.

It is possible that the loss of neurofibromin in Schwann cells induces the aberrant expression of c-kit. However, the molecular mechanism for the abnormal expression of this growth factor receptor is still under investigation. It may be possible that c-kit may be a receptor involved in the development of Schwann cells and due to a series of mutations, including the loss of neurofibromin, c-kit is inappropriately re-expressed in NF1 Schwann cells. In addition, c-kit can be activated by mast cells which are able to secrete SCF (DePaulis et al., 1999).

Interestingly, Zhu and colleagues (2002) reported a great number of mast cells within tumors of Nf1^{+/-} mice suggesting that they may provide a favorable environment for the development of neurofibromas. These results are consistent with the view that increased survival and proliferation of Schwann cells contribute to the formation of NF1 tumors.

The expression of c-kit has been associated with several types of tumors including gliomas, melanomas, myeloid leukemias, small cell lung cancer, and breast cancer (Tuner et al., 1992; Krystal et al., 1996). In humans, series of gain-of-function mutations in the c-kit juxtamembrane region have been found in gastrointestinal stromal cell tumors (Hirota et al., 1998). C-kit is also aberrantly expressed in approximately 70% of all small cell carcinomas in the lung (SCCL), as well as in breast, cervical, and ovarian tumors (Tuner et al., 1992; Hines et al., 1995; Krystal et al., 1996). Schwann cells derived from NF1 tumors have high levels of c-kit expression contributing to Schwann cell neoplasia (Badache et al., 1998). Coexpression of c-kit and SCF in NF1 Schwann cells generates an autocrine loop that may play a role in the etiology of NF1 (Badache et al. 1998; Ryan et al., 1994). Our findings suggest that additional cell lines, the 90-8 and 88-3, express c-kit as well. In contrast to NF1 Schwann cells, normal adult human Schwann cells expressing neurofibromin do not have c-kit on their surfaces. Taken together, the loss of neurofibromin in Schwann cells may induce aberrant expression of growth factor receptors, such as c-kit, contributing to Schwann cell transformation and the development of NF1 tumors.

C-kit expression has been detected in many glial cells, but the normal function of the c-kit receptor differs depending on the cell type. For instance, high c-kit expression levels induced by HIV are associated with astrocyte apoptosis (He et al., 1997). In the case of oligodendrocytes, c-kit expression is increased between postnatal days 10 and 12, suggesting that

c-kit and its ligand may play a role during oligodendrocyte development (Ida et al., 1993).

Similarly, c-kit expression is developmentally regulated in Schwann cells. Our results suggest that activation of c-kit prevents Schwann cell apoptosis in the embryonic and neonatal stages of development in order to obtain the appropriate Schwann cell numbers needed for the myelination of a segment of an axon. Previous reports have shown that the ligand for c-kit, SCF, may be secreted by axons (Young et al., 1998) or may also be secreted from mast cells (De Paulis et al., 1999). Taken together, SCF may be a growth factor important for the survival of Schwann cell during development.

Although we do know the exact functions of neurofibromin, an inverse correlation exists between neurofibromin and c-kit expression. The loss of neurofibromin expression is the first event in a long series of events that leads to the re-expression of c-kit, which contributes to the increased survival and proliferation of NF1 Schwann cells. These results may provide a better understanding of the molecular mechanisms leading to the abnormal formation of tumors in NF1 patients.

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Figure Legends

Figure 1. Developmental analysis of c-kit, P0, and neurofibromin protein in rat sciatic nerves. Rat sciatic nerves were homogenized with lysis buffer, and 100ug of proteins were analyzed by immunoblotting with c-kit (A), P0 (B), or neurofibromin (C) antibodies. These immunoblots are a representative experiment repeated 3 times with similar results.

Figure 2. C-kit and neurofibromin expression in Schwann. Cells were lysed and 50ug of proteins were analyzed by immunoblotting with c-kit (A) and neurofibromin (B) antibodies. These immunoblots are a representative experiment repeated 3 times with similar results.

Figure 3. SCF induces weak ERK phosphorylation but strong Akt phosphorylation in neonatal rat Schwann cells. Schwann cells were cultured with 50ng/ml of SCF for up to 120 minutes. Schwann cells were lysed and 25ug of proteins were analyzed by immunoblotting with a phosphorylated specific Akt antibody (panel A) and the phosphorylated specific ERK antibody (panel B). These immunoblots are a representative experiment repeated 3 times with similar results.

Figure 4. SCF does not induce neonatal rat Schwann cell proliferation or differentiation. Schwann cells were cultured in the presence of increasing concentrations of SCF alone or with 20ng/ml β 1 neuregulin for 96 hours (A). Cell number was evaluated by the colorimetric MTT assay after 96 hours in culture. Values are expressed as mean of at least 3 replicates from a representative experiment repeated 3 times. Error bars represent standard deviations. The data were statistically analyzed using One Way Analysis of Variance (ANOVA) and no statistical significance was found for SCF alone ($F=0.990$) or in the presence neuregulin ($F=0.962$). Differentiating Schwann cells were assayed by RT/PCR for P0 mRNA (C). Schwann cells were cultured in the presence of 100 ng/ml of SCF up to 72 hours. After RNA extraction, RT/PCR reactions were performed for each time point. Photograph of the P0 products, 603 base pairs, were separated on a 2% agarose gel stained with ethidium bromide. Molecular weight markers (MW) are derived from Hae III digest of ϕ X-174 viral DNA. These PCR products are a representative experiment repeated 3 times with similar results.

Figure 5. SCF promotes Schwann cell survival. Schwann cells were cultured in the presence of SCF for 72 hours. Using the TUNEL assay, apoptotic cells display fluorescent nuclei. Control cells without TdT (A), serum free medium (B), Schwann cells in serum free medium with SCF (50ng/ml) (C), and Schwann cells in serum free medium with neuregulin (50ng/ml) (D). These photographs are a representative field of an experiment for repeated 3 times with similar results. The graph summarizes the results of apoptotic assays of Schwann cells cultured in serum free media, in the presence of SCF (25 or 50ng/ml), or in the presence of SCF (50ng/ml) with LY294002 (10uM) for 72 hours (E). Apoptotic cells were determined using TUNEL assays. Values are the mean percentage of apoptotic cells of an experiment repeated 3 times. Error bars represent +/- standard error of the mean. 2 fields of 120-150 cells were counted for each experiment at each condition. The data were determined to be statistically significant using ANOVA ($F(3,8)=13.992$). Post-hoc comparisons were determined using Tukey's LSD (*, significantly different from control, $P<0.05$). Bar is 10 uM.

Figure 1

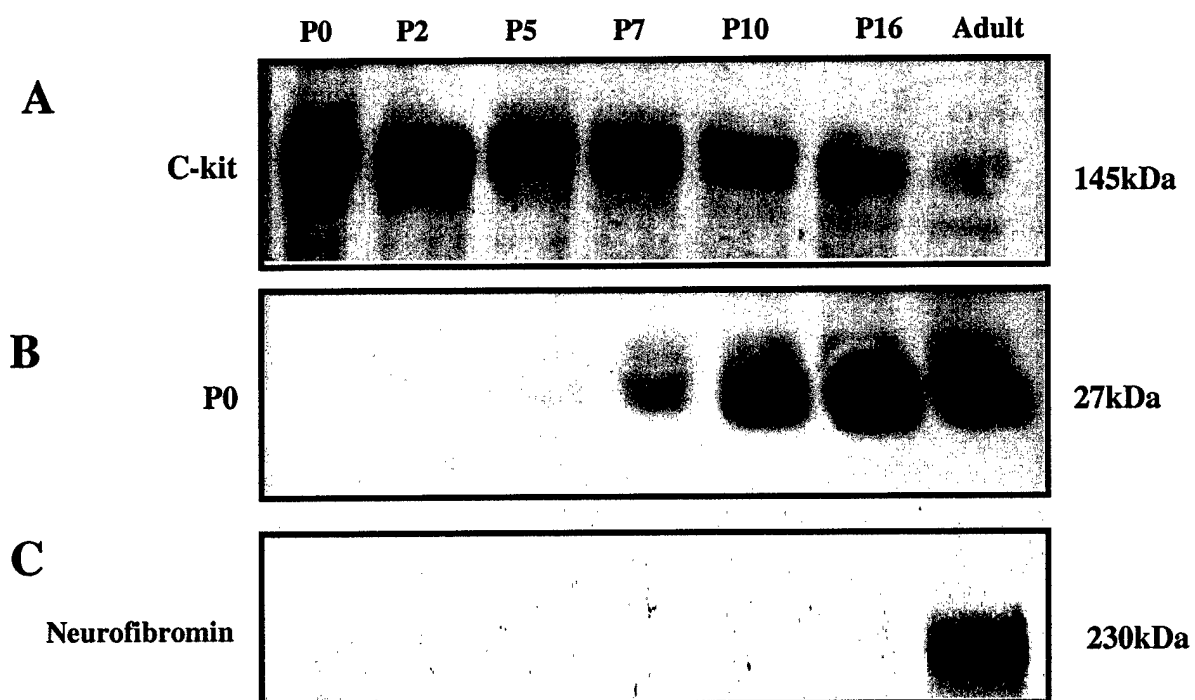


Figure 2

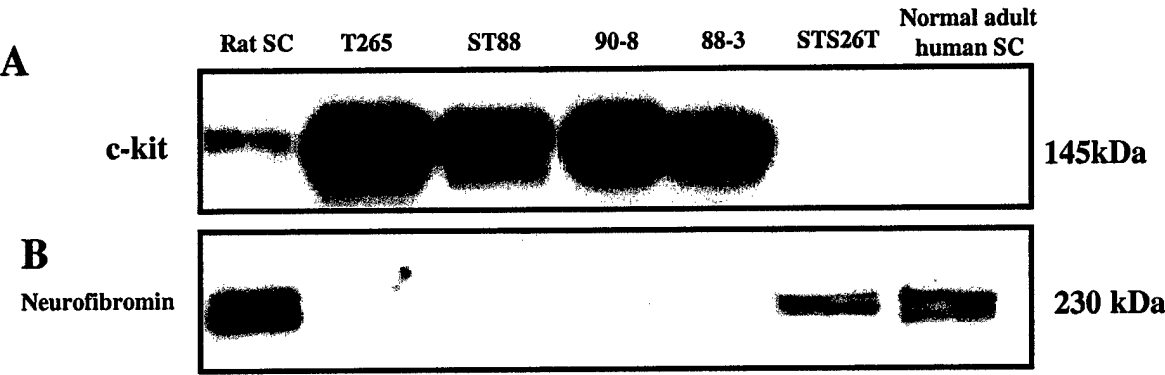


Figure 3

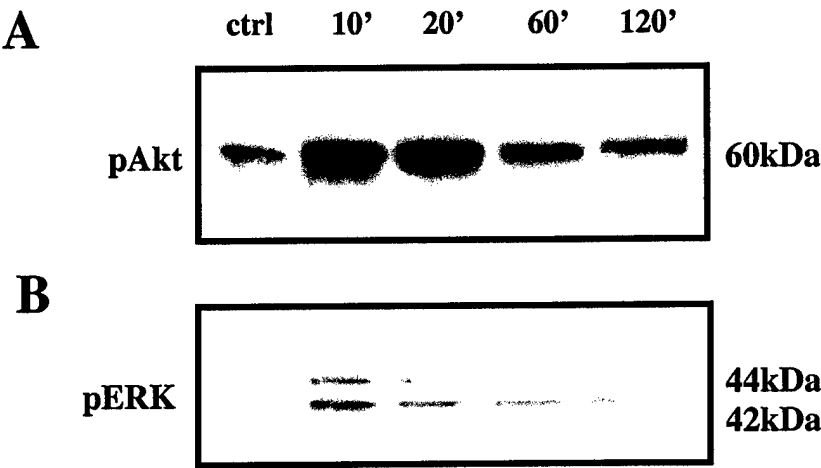


Figure 4

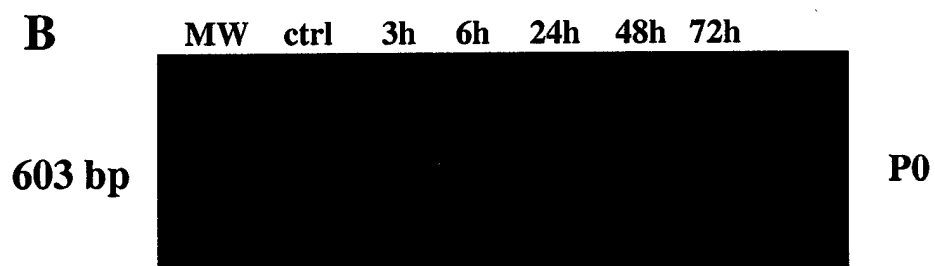
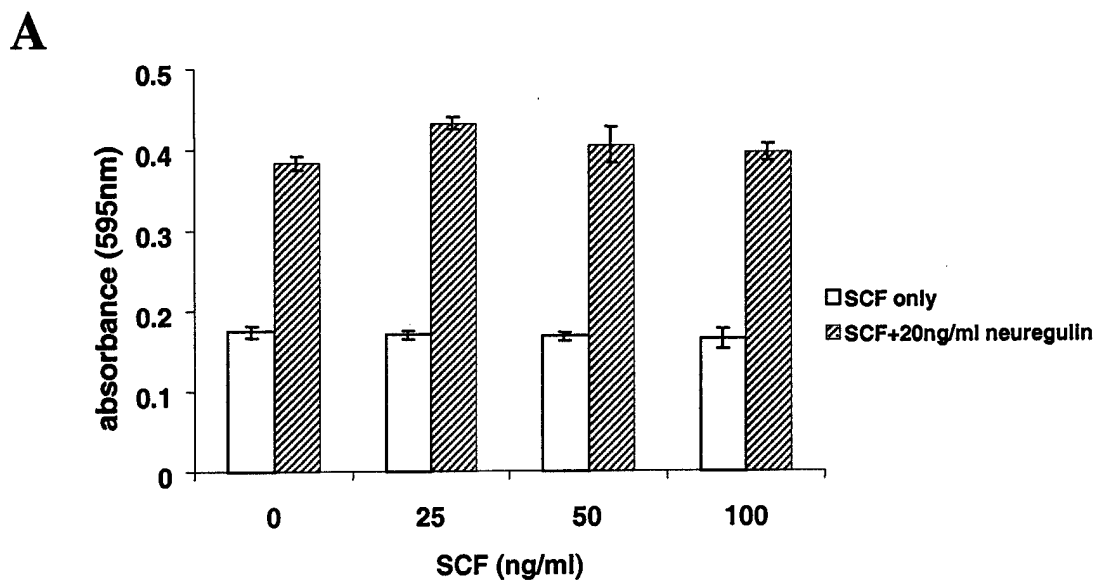


Figure 5

